AD_____

Award Number: DAMD17-01-1-0689

TITLE: The BESCT Lung Cancer Program (Biology, Education, Screening, Chemoprevention, and Treatment)

PRINCIPAL INVESTIGATOR: Waun K. Hong, M.D.
Fadlo R. Khuri, M.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

REPORT DATE: April 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-04-2007	Annual	15 Mar 2006– 14 Mar 2007

4. TITLE AND SUBTITLE

The BESCT Lung Cancer Program (Biology, Education, Screening, Chemoprevention, and Treatment)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0689

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Waun K. Hong, M.D.
Fadlo R. Khuri, M.D.

Email: whong@mdanderson.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

BESCT program aims to define molecular abnormalities contributing to lung cancer initiation and progression and to develop innovative therapeutic approaches for this cancer. Our specific aims are 1) to understand molecular alterations in lung cancer, 2) to develop chemoprevention strategies for lung cancer, and 3) to implement experimental molecular therapeutic approaches for lung cancer treatment.

15. SUBJECT TERMS
Lung Cancer, genetic alterations, chemoprevention, molecular therapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	177	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Lung cancer is a devastating public health hazard worldwide. Advances in surgery, radiation therapy, and chemotherapy of non-small cell lung cancer (NSCLC) have only led to a marginal improvement in five-year survival. To reduce lung cancer incidence and mortality, it is imperative to develop effective therapeutic and preventive strategies targeting lung cancer patients. The BESCT program developed in 2001 aims to define molecular abnormalities contributing to lung cancer initiation and progression and to develop innovative therapeutic approaches for this cancer. Our specific aims are as follows:

- To understand molecular alterations in lung cancer
- To develop chemoprevention strategies for lung cancer
- To implement experimental molecular therapeutic approaches for lung cancer treatment

BESCT is composed of 3 research projects. This report describes the progress for the sixth grant year (March 15, 2006 to March 14, 2007), year 1 of a 2-year non-funded extension from the Department of Defense. This extension was requested due to unforeseen delays opening the BESCT clinical trial to be conducted at Emory University and to complete remaining Specific Aims.

PROGRESS REPORT (Body)

Project 1: Study Mechanisms of Molecular Alterations in Lung Cancer

(PI: Li Mao, M.D.)

Specific Aim 1 ***To determine the mRNA complex responsible for C-CAM1 splicing and identify factor(s) regulating exon 7 splicing.***

This Aim was completed as reported in 2005. From this research, an extended Aim (Aim 6) was developed and will be covered below.

Specific Aim 2 ***To determine function of identified splicing factor(s) in regulation of CEACAM1 and its potential alterations in lung cancer.***

This Aim was summarized and completed in 2005. A manuscript (Wang et al., 2007) is in preparation.

Specific Aim 3 ***To determine function of DNA methyltransferases and their role in controlling methylation and expression of critical tumor suppressor genes and tumor antigen genes.***

This Aim was completed in 2004. Two publications have been generated from this research, one in *Clinical Cancer Research* (Wang et al., 2004) and the other in *Molecular Cancer Research* (Tang et al., 2004), as reported previously.

Specific Aim 4 ***To determine expression and abnormalities of DNMT3B isoforms in lung tumorigenesis and their association with de novo DNA methylation patterns, and clinical applications.***

Update

In the last report, we reported the analysis of the expression of seven splice variants of DNMT3B in 119 primary non-small cell lung cancer (NSCLC) specimens and their corresponding non-

malignant lung tissues using specific primer sets. We found that *DNMT3B* variants are involved in differential regulation of promoter methylation in lung tumorigenesis and may serve as biomarkers in early detection of lung cancer and for molecular classification.

In the past year, we have identified *ΔDNMT3B* as the major expression form of DNMT3B in lung cancer and the relationship between the expression of *ΔDNMT3B* variants and certain promoter methylation. We further examined their relationship in different cell lines. We found that inactivation of *ΔDNMT3B2/4* variants is critical in methylation of certain promoters such as RASSF1A and DAP-kinase in lung cancer cells but not in colorectal or in prostate cancer cells. The inactivation of *ΔDNMT3B2/4* variants also results in increased apoptosis in lung cancer cells.

Based on these findings, we published two manuscripts, one in *International Journal of Oncology* (Wang and Wang et al., 2006) and the other in the *Cancer Research* (Wang and Walsh et al., 2006) (Appendix A – Publications). In addition, we filed for a U.S. patent for this discovery (Patent application No. 20060115829).

This aim has been completed.

Specific Aim 5 ***To determine expression of hnRNP-A1 variants in lung cancer cells and their role in the regulation of pre-mRNA splicing.***

Limited progress has been made for this task due to our inability to isolate the protein complex responsible for the splicing abnormality. Thus, this task was terminated and our emphasis was shifted to the extended task, Specific Aim 6, as reported last year.

Specific Aim 6 ***To determine the role of hepatoma-derived growth factor (HDGF) in lung cancer.***

Update

In the past year, we have made significant progress for this Aim which is summarized below.

To determine the HDGF function in lung cancer progression, we knocked down HDGF with *HDGF*-specific small interfering RNA (siRNA) in four NSCLC cell lines. Down-regulation of HDGF didn't affect cell proliferation and cell cycle as detemined by MTT assay and flow cytometric analysis, but cells transfected with *HDGF*-siRNA grew more slowly and formed significantly fewer colonies in soft agar than with Lipofectamine alone or negative control siRNA. In an *in vitro* invasion assay, fewer cells with *HDGF*-siRNA invaded across a Matrigel membrane barrier than the controls. In an *in vivo* mouse model, *HDGF*-siRNA-transfected A549 cells grew significantly more slowly than the control cells. Further, *HDGF*-siRNA treated tumors exhibited markedly reduced blood vessel formation and increased necrosis; whereas, their Ki67 labeling indices were similar to those of the control tumors, consistent with our *in vitro* data. Thus, the results suggest that HDGF is involved in anchorage-independent growth, cell invasion, and formation of neovasculature of NSCLC. For detailed findings, please review our publication in *Cancer Research* (Zhang et al., 2006) (Appendix A – Publications).

Based on the *in vitro* and *in vivo* data, we have proposed that HDGF is a potential therapeutic target for cancer. Therefore, we have begun development of therapeutic application(s) of HDGF. One strategy is to develop neutralizing antibodies. We first used recombinant HDGF to develop a panel of monoclonal antibodies that specifically bind to HDGF, 2 of which (C1 and H3) exhibited significant antitumor activity in A549 lung cancer xenografts. H3 is also effective in MiaPaca-2 pancreatic cancer xenografts. No toxicity was observed in the living mice and major organs of antibody-treated mice. In the A549 xenografts, the mean tumor burden was 960 mm^3 for control

IgG-treated mice 22 days after tumor inoculation, whereas the mean tumor burdens were 224 mm^3 for C1- and 266 mm^3 for H3-treated mice ($P < 0.05$), respectively. In the MiaPaca-2 xenografts, the mean tumor burden was 994 mm^3 for control IgG - treated mice 21 days after tumor inoculation in comparison to 345 mm^3 for H3-treated mice ($P < 0.05$). Consistent with known biologic functions of HDGF, our morphologic and biomarker analyses suggest that H3 may neutralize HDGF released from tumor cells, resulting in disruption of tumor stroma and extracellular matrix structures. Thus, we conclude that HDGF is a novel therapeutic target for multiple human cancers and a neutralized monoclonal antibody targeting HDGF will be effective in treating lung and pancreatic cancers.

Based on these findings, we have published two manuscripts, one in the *Journal of Clinical Oncology* (Ren, et al., 2004), and the other in *Cancer Research* (Zhang et al., 2006) as mentioned above. We also filed for a U.S. and worldwide patent for the discovery in December 2006 (Patent Application No. 11611554 and International Patent Application No. PCT/US2006/062176) (Mao et al., 2006). This technology has been licensed to PDL Biopharma (Fremont, CA) for further development towards clinical applications, particularly in the treatment of lung cancer patients.

This Aim has been completed.

Key Research Accomplishments:

- Demonstrated that HDGF involves anchorage-independent growth, invasion, and neovasculature formation of NSCLC cells during lung cancer progression.
- Demonstrated HDGF as a novel therapeutic target for lung and other major cancer types.
- Developed monoclonal antibodies against HDGF as potential therapeutic agents and demonstrated their therapeutic efficacies in treating lung and pancreatic cancers in tumor xenograft models.
- Successfully licensed the HDGF technology to a major pharmaceutical company for further development towards potential clinical applications.
- Discovered an important role of ΔDNMT3B variants in differential regulation of promoter DNA methylation, which may lead to the development of novel cancer therapeutic strategies.

Reportable Outcomes:

Publications in peer-reviewed journals

- Wang J, Walsh G, Liu D, Lee JJ, Mao L. Expression of *ΔDNMT3B* Variants and its association with promoter methylation of *p16* and *RASSF1A* in primary non-small cell lung cancer. *Cancer Res*, 66:8361-8366, 2006.
- Wang L, Wang J, Sun SY, Rodriguez M, Yue P, Jang SJ, Mao L. A Novel DNMT3B Subfamily, ΔDNMT3B, is the Predominant Form of DNMT3B in Non-Small Cell Lung Cancer. *Internatl J Oncol*, 29:201-207, 2006.
- Zhang J, Ren H, Yuan P, Lang W, Zhang L, Mao L. Down-Regulation of Hepatoma-Derived Growth Factor Inhibits Anchorage-Independent Growth and Invasion of Non-Small Cell Lung Cancer Cells. *Cancer Res* (Priority report) 66:18-23, 2006.

Abstracts

- Mao L, Ren H, Chu Z, Yuan P. Therapeutic activity of neutralizing monoclonal antibodies targeting hepatoma-derived growth factor in cancer xenograft models. *ASCO Annual Meeting Proceedings* 42, Abstract #: 2517, 2006.
- Zhang J, Mao L. SiRNA targeting hepatoma-derived growth factor (HDGF) inhibits growth of non-small cell lung cancer in xenograft models. *Proceedings of AACR Annual Meeting*, 2006, Abstract #5135.

Patents

- Mao L et al. Anti-Hyperproliferative Therapies Targeting HDGF. Patent application No. 11611554; International Patent Application No. PCT/US2006/062176, filed December 15, 2006.
- Mao L et al. Method of treating a cancer. Patent application No. 20060115829.

Conclusion and Future Study:

We have demonstrated the importance of ΔDNMT3B, a novel subfamily of DNMT3B discovered in the project, in lung tumorigenesis. More importantly, we have showed that ΔDNMT3B variants play an important role in regulation of promoter methylation in a gene-specific manner. This discovery has potentially significant implication in development of novel cancer therapies by regulating gene expression through modulating levels of individual deltaDNMT3B variants. These studies highlight the critical role of alternative splicing of pre-mRNA in lung tumorigenesis.

The discovery of HDGF as a key factor in lung cancer progression provides a potential biomarker to predict patients' outcomes and a potential target for treating lung cancer. A major success of the project is the development of candidate therapeutic monoclonal antibodies. We have demonstrated that these antibodies are effective in inhibiting lung cancer growth *in vivo* animal models through novel mechanisms. We have reached a major milestone by licensing the technology to a major biopharmaceutical company for antibody humanization and clinical development, a step critical to translate our laboratory research discoveries to patient care.

Proposed aims have been accomplished and the study is closed.

Project 2: Develop Novel Strategies for Lung Cancer Chemoprevention
(Project Leader: Fadlo Khuri, M.D.)

Specific Aim 2.1 To evaluate the effects of oral bexarotene delivered to former smokers by inhalation alone or in combination with celecoxib.

(PI: Fadlo Khuri, M.D)

As reported in 2004, we were unable to conduct the clinical trial proposed in the original Specific Aim, "To evaluate the effects of aerosolized 13cRA delivered to former smokers by inhalation alone or in combination with Celecoxib," due to excessive toxicity with aerosolized delivery. Initial clinical studies have indicated that retinoids decrease the incidence of second primary tumors in patients who have previously undergone resection for NSCLC or head and neck cancer. However, subsequent large-scale chemoprevention trials have demonstrated that retinoids induce substantial toxicity and are of minimal benefit to individuals at high-risk for lung cancer, illustrating the need for more effective lung cancer chemoprevention strategies.

We have thus proposed another clinical trial "A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients With Solid Tumors Previously Treated With Standard Chemotherapy." The objective of this project is to evaluate the chemopreventive potential of bexarotene (Targretin®) and non-steroidal anti-inflammatory agents (NSAIDs) (e.g., celecoxib) in high-risk lung cancer patients.

Update

The protocol was reviewed and approved by Emory Institutional Review Board (IRB) on January 5, 2007 (Appendix B-C - Project 2.1-Emory IRB approved protocol and approval letter). Recently, we have received the final revisions from the February 14, 2007, DoD-HSRRB (Human Subjects

Research Review Board), and returned the responses to the DoD on March 14, 2007. We also have concluded an agreement with Eisai Pharmaceuticals regarding the supply of Targretin. We expect to have the trial opened in a few weeks.

Specific Aim 2.2 ***To evaluate effects of NSAIDs and 4HPR (replace 13cRA) as single agents and in combinations on growth, apoptosis, and carcinogenesis using an in vitro cell system and an animal model.***

(PI: Reuben Lotan, Ph.D.)

Because the retinoid 13cRA shows enhancement of lung cancer among smokers in a clinical trial but has side effects, whereas the synthetic retinoid fenretinide [*N*-(4-hydroxyphenyl) retinamide, 4HPR] is more potent with fewer side effects and has additive effects with celecoxib, we have replaced 13cRA with 4HPR for subsequent *in vitro* and animal experiments.

Update

As reported last year, the combination of the COX-2 inhibitor, celecoxib, and 4HPR enhanced growth inhibition and apoptosis induction in NSCLC cell lines and premalignant lung epithelial cell lines. The results were published in *Cancer Biology & Therapy* (Sun et al., 2005) and *Cancer Research* (Schroeder et al., 2006).

We have expanded these observations to metabolites of arachidonic acid-metabolizing enzymes, cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX), 5-LOX-activating protein (FLAP) and their products (e.g., PGE_2, 5-HETE, and LTB_4), which are increased during lung carcinogenesis. We found that simultaneous treatment of premalignant and malignant human lung cell lines with the COX-2 inhibitor, celecoxib, and the 5-LOX inhibitors, MK886 and REV5901, is more potent in growth suppression and induction of cell death than single or dual combinations of these agents. However, their sensitivity to the inhibitors was not directly associated with the expression of COX-2, 5-LOX, or FLAP, but correlated with the production of corresponding metabolites. Furthermore, partial protection of cell death was observed when PGE_2 and/or 5-HETE was added to cell cultures treated with celecoxib, MK886, and REV5901 simultaneously. These results have been summarized in a manuscript that is in press (Schroeder et al., 2007).

The animal experiments with 4HPR have been completed, and the data with celecoxib are currently being analyzed by the Pathologist. Gprc5a(-/-) mice were exposed to the tobacco carcinogen NNK injected i.p. in 2-3 month old mice. One month later, the mice were randomized into two groups, one for a control diet and the other for a diet with 4HPR (2 mmol/Kg diet). Fifteen months later, the mice were killed; their lungs were perfused with formalin and analyzed for the presence and number of tumors. We found that the incidence of lung tumors was 75% (12/16) and 53.3% (8/15) in mice on control diet and 4HPR-supplemented diet, respectively. The formalin fixed lungs were embedded in paraffin and used to prepare histological sections. Some sections were stained with hematoxylin and eosin to identify and quantify lung adenomas and adenocarcinomas. The incidence of adenomas in control and 4HPR treatment groups was 5/16 and 6/15, respectively, and the incidence of adenocarcinoma in these groups was 43.8% (7/16) and 13.3% (2/15), respectively. The mean number of tumors per mouse (MNT) that had developed between the control and 4HPR groups for adenocarcinoma alone was 0.5 and 0.2, respectively, and was borderline-significant ($p=0.068$). Other sections were used for immunohistochemical analyses of microvessel density (CD31), proliferation markers (Ki67 and cyclin D1), and apoptosis (cleaved capsase 3). The results in Figure 1 show that 4HPR caused decreased microvessel density and proliferation and an increased apoptosis.

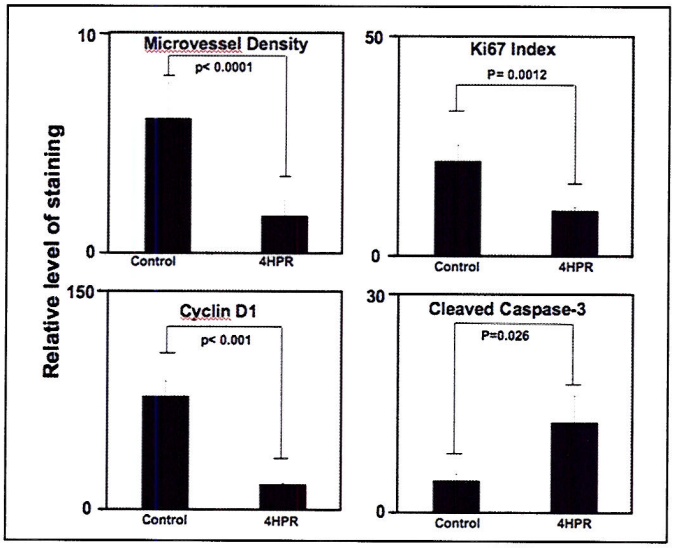


Figure 1. Histological sections of tumors from lungs of control group on regular diet and treatment group on diet supplemented with 4HPR were analyzed by immunohistochemistry using antibodies against the endothelial cell marker CD31, proliferation markers Ki67 and cyclin D1, and the apoptosis marker cleaved caspase 3. Two microscopic fields at magnification X200 were analyzed for percentage of positive cells multiplied by the intensity of staining on a scale of 1 to 3.

Specific Aim 2.3. ***To investigate whether genetic approaches to inhibit PI3K activity decrease lung tumor size and number in k-ras mutant mice.***

(PI: Ho-Young Lee, Ph.D.)

This Aim has been discontinued as reported in the 2004 Annual Report due to the variability in the delivery system. A more reliable system was developed and, thus, pursued through our Lung Cancer SPORE grant.

Specific Aim 2.4. ***To analyze differential gene expression between untreated NSCLC cells and celecoxib-treated NSCLC cells using affymetrix oligonucleotide microarrays and characterize genes that may be implicated in mediating apoptosis induction.***

(PI: Reuben Lotan, Ph.D.)

Update

Now that we have completed the animal experiments described above, we will begin this aim right away.

Key Research Accomplishments:

- Completed revisions of the protocol for the Phase I combination trial of bexarotene and celecoxib in solid tumors. Received the approval from the Emory IRB on January 5, 2007, and awaiting final approval from the DoD.
- Found that the sensitivity to the Cox-2 or 5-Lox inhibitors was not directly associated with the expression levels of COX-2, 5-LOX, or FLAP, but was correlated with the production of the corresponding metabolites.
- Demonstrated that 4HPR exerts a borderline significant suppression of the development of lung adenocarcinomas in NNK-exposed Gprc5a knockout mice. The anti-angiogenic and growth inhibitory plus pro-apoptotic effects of 4HPR could account for its chemopreventive activity.

Reportable Outcomes:

Publications in peer-reviewed journals

- Schroeder CP, Kadara H, Lotan D, Woo JK, Lee HY, Hong WK, Lotan R. Involvement of Mitochondria and Akt-Signaling Pathway in Augmented Apoptosis Induced by a Combination of

Low Doses of Celecoxib and 4HPR in Premalignant Human Bronchial Epithelial Cells. *Cancer Res,* 66: 9762-70, 2006.

- Schroeder CP, Yang P, Newman RA, Lotan R. Simultaneous inhibition of COX-2 and 5-LOX activities augments growth arrest and death of premalignant and malignant human lung cell lines. *J. Exptl. Therap. Oncol,* In press.

Conclusions:

Our data suggest that the combination of celecoxib and 4HPR or combinations of celecoxib and LOX inhibitors may be useful for lung cancer chemoprevention and treatment.

Project 3: Implement Experimental Molecular Therapeutic Approaches for Lung Cancer
(Project Leader: Fadlo Khuri, M.D.)

Specific Aim 3.1 ***To develop a relatively faithful murine model of lung cancer by crossing the k-ras mutant mouse (T. Jacks) with p53 mutant missense mouse (G. Lozano) and study the evolution of non-small cell lung cancer in primary lung tumor model with metastatic potential and the effectiveness of targeted agents in the model.***

(PI: Guillermina Lozano, Ph.D.)

Update

This Aim was completed in 2005. A manuscript titled "A Genetic Mouse Model for Metastatic Lung Cancer with Gender Differences in Survival" was submitted to *Oncogene* for review (Zheng et al., 2007).

Mice inheriting both *p53* and *K-ras* missense mutations develop tumors that progress from adenomas to invasive adenocarcinomas. We have now asked whether mice inheriting *K-ras* and *p53* mutations could serve as a model for preventive and therapeutic studies as an extended Task (see Specific Aim 3.5).

Specific Aim 3.2 ***To evaluate novel signal transduction inhibitors alone, in combination with one another, or with cytotoxic agents in the treatment of the mouse lung cancer and, ultimately, in the treatment of human lung cancers.***

(PI: Fadlo Khuri, M.D.)

Update

We have studied farnesyltransferase inhibitors (FTIs) including lonafarnib in the past five years and found that lonafarnib increased microtubule acetylation and synergy with taxanes in anti-proliferation activity. Detailed findings were published in *Cancer Research* (Marcus et al., 2005). If indeed synergy between taxanes and lonafarnib is achieved at low doses and is manifested by an increase in microtubule acetylation, this is a hypothesis that needs to be tested prospectively.

We therefore designed a Phase Ib combination trial of lonafarnib and docetaxel in biopsy-accessible solid tumor patients to combine lonafarnib with low doses of docetaxel weekly as an extended study. We plan to continue collaborations with Adam Marcus, PhD, Instructor of Hematology & Oncology, Winship Cancer Institute, and Paraskevi Giannakakou, Ph.D. (now Associate Professor of Medicine and Pharmacology at Cornell University) to further explore the

mechanism of this interaction while we initiate this Phase Ib protocol with serial biopsies to assess the efficacy of this combination in various solid tumor patients.

The trial is supported by our NIH/NCI P01 (CA116676-01A1) Program Project grant and Sanofi-Aventis pharmaceuticals, and lonafarnib is provided by Schering Plough. This trial is activated and has 12 patients accrued thus far.

For additional studies of other targeted agents, please refer to Specific Aim 3.5 below.

Specific Aim 3.3 ***To produce and test a liposomal gene-therapeutic strategy targeted to a novel tumor suppressor gene located on chromosome 3p, both in the mouse model and in human patients with advanced non-small cell lung cancer.***

(PI: Charlie Lu, M.D)

Update

As reported in 2005, we have completed the preclinical study, and the preclinical data were used to develop the phase I trial of DOTAP:cholesterol-FUS1 liposome complex (Human Gene Transfer Protocol #0201-513) that is supported by different financial agencies, and thus the status of the trial doesn't require to be included in the Annual report, but we do like to share some progress of the trial as follows.

The Phase I trial in advanced NSCLC patients has been conducted at M. D. Anderson Cancer Center. Between May and September 2003, six patients were enrolled and received a total of 15 treatment cycles (range 1 to 6 cycles). Because of toxicity issue, the trial was placed on a clinical hold for FDA review between November 2003 and February 2004. After reopening the protocol, the first cohort of 3 patients was enrolled between 8/2004 and 1/2005 and was treated at a reduced starting dose level of 0.01 mg/Kg without the use of premedications. 2 of 3 patients developed evidence of an inflammatory response characterized by fever (grade 3), chills, and hypotension (grades 2 and 3). After consulting with the FDA, the protocol was amended to require steroid premedication. Treatment with premedication was begun at the same initial dose level of 0.01 mg/Kg. Subsequently, the M. D. Anderson Institutional Compliance Office reviewed the protocol and made a decision to transfer the manufacture of the DOTAP:cholesterol-FUS1 liposome complex from a laboratory facility at Baylor College of Medicine to M. D. Anderson Cancer Center's GMP facility. The study drug has been successfully produced at the M.D. Anderson facility, and the trial was reopened last year.

To date, 13 patients have been entered on the study at three different doses (0.1, 0.02, 0.03mg/kg). All patients are evaluable for the primary endpoint of toxicity, and with steroid premedications, there have been no significant drug related toxicity. Four patients received only one dose because of rapidly progressing disease at a site requiring local treatment. Eight patients received two or more doses and are evaluable for response, with 3 patients achieving stable disease (3 - 7 months) and 5 patients with progressive disease. Median survival time for these 13 patients is 14.6 months, which compares favorably to the published 7 months median survival for the second line chemotherapy. A maximum tolerated dose (MTD) has not been reached. Enrollment to the trial continues.

Specific Aim 3.4 ***To develop specific vascularly targeted strategies to the vascular endothelium of lung cancer cells to decrease the toxicity to normal cells and enhance the therapeutic index.***

(PI: Ho-Young Lee, Ph.D)

In the last report, we showed that the farnesyl transferase inhibitor (FTI), SCH66336, has antiangiogenic activity in aerodigestive tract cancer, including lung and head/neck cancers, by suppressing the expression of HIF-1α and VEGF (Han et al., 2005). We also demonstrated that the insulin growth factor 1(IGF)-mediated pathway plays an important role in angiogenesis by stimulating the synthesis of HIF-1α and VEGF, and its down-stream signaling mediators, including phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK), cooperate to promote cell proliferation. Thus, we have tested whether inhibition of PI3K/Akt and MAPK pathways effectively inhibits NSCLC tumor growth and angiogenesis.

We have found that intratumoral injection of adenoviruses expressing a dominant-negative mutant of the p85α adaptor subunit of PI3K (Ad-dnp85α) induced a significant decrease in growth of H1299 NSCLC xenograft tumors. Concurrent inhibition of the PI3K/Akt and MKK4/JNK pathways by injecting adenoviruses expressing the dominant-negative Akt [Ad-HA-Akt(KM)] and the dominant-negative MKK4 [Ad-MKK4(KR)] showed enhanced antitumor effects on H1299 xenograft tumors by inducing apoptosis and inhibiting angiogenesis (Lee et al., 2005). The results indicate that PI3K/Akt and MKK4/JNK pathways cooperate to stimulate cell proliferation and angiogenesis, suggesting that simultaneously targeting the two pathways might be an effective therapeutic strategy against NSCLC.

Although we have demonstrated that the FTI SCH66336 has apoptotic and antiangiogenic activities in aerodigestive tract cancer, including lung and head/neck cancers (Han et al., 2005), clinical trials have shown a limited antitumor activity in cancer patients (Morgillo et al., 2006-review) (Appendix A – Publications), and the mechanism of the resistance to FTI treatment is unknown.

Update

In the past year, we investigated the mechanisms of resistance to SCH66336 in aerodigestive tract cancer using lung and head/neck cancer cell lines.

As we reported, a subset of head and neck squamous carcinoma cell (HNSCC) cell lines, SqCC/Y1, TR146, UMSCC38, UMSCC22A, 183A, and UMSCC17B, have shown apoptotic response when treated with SCH66336 in 5% serum for 5 days (Chun et al., 2003). However, we have recently observed a rather large differential response of some HNSCC cell lines to SCH66336 (Figure 1*B*). SqCC/Y1 (Figure 1A) and SqCC35 (data not shown) remained sensitive to apoptotic activities of SCH66336 in

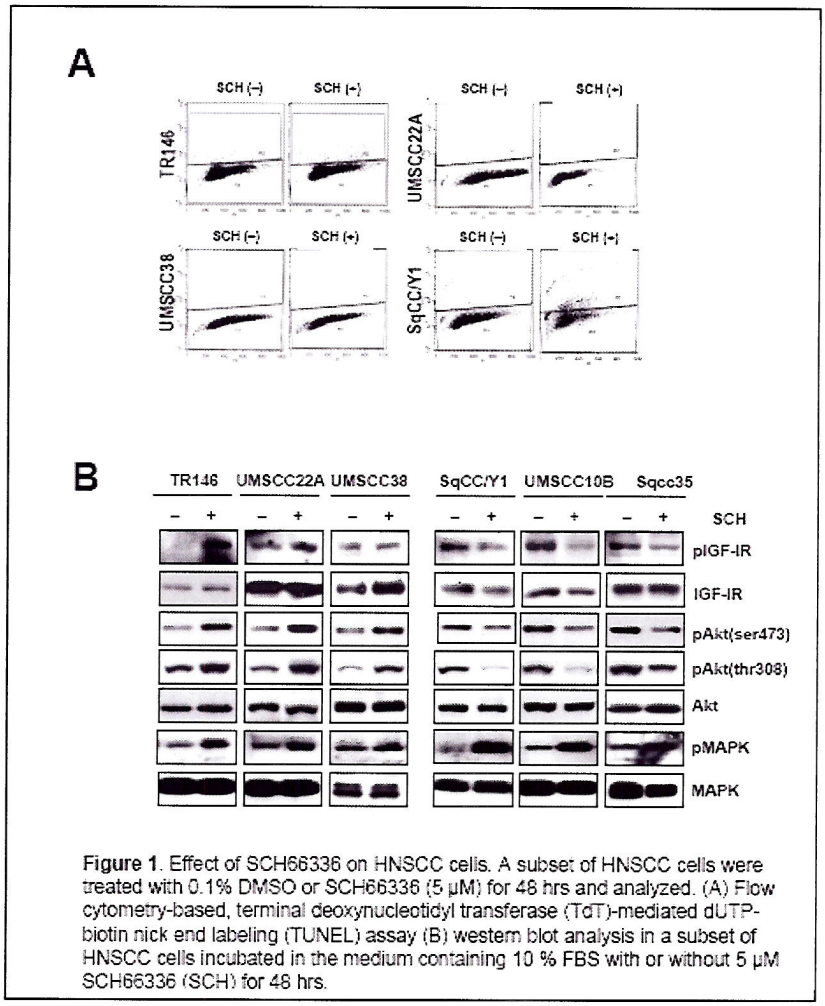


Figure 1. Effect of SCH66336 on HNSCC cells. A subset of HNSCC cells were treated with 0.1% DMSO or SCH66336 (5 μM) for 48 hrs and analyzed. (A) Flow cytometry-based, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay (B) western blot analysis in a subset of HNSCC cells incubated in the medium containing 10 % FBS with or without 5 μM SCH66336 (SCH) for 48 hrs.

the presence of 10% serum. In contrast, including TR146, UMSCC22A, and UMSCC38 cells showed no evidence of apoptosis in the same condition (Figure 1*B*).

Because activation of IGF-1R and consequent activation of IRS-I and Akt have been known to mediate resistance to antitumor agents in variable cancers, we examined the status of IGF-1R/Akt signaling pathway in the HNSCC cells after the treatment with SCH66336. We found that IGF-1R and Akt were activated in the HNSCC cell lines insensitive to apoptotic activities of SCH66336 (TR146, UMSCC22A, and UMSCC38) after the treatment with SCH66336 in 10% FBS.

To confirm the role of IGF-1R in the resistance to apoptosis induced by SCH66336, TR146 and UMSCC38 cells were infected with adenovirus expressing the dominant negative IGF-1R (Ad-dn IGF-1R) (Figure 2*A*) or treated with IGF-1R tyrosine kinase inhibitor AG1024 (Figure 2*B*) prior to the incubation with SCH66336.

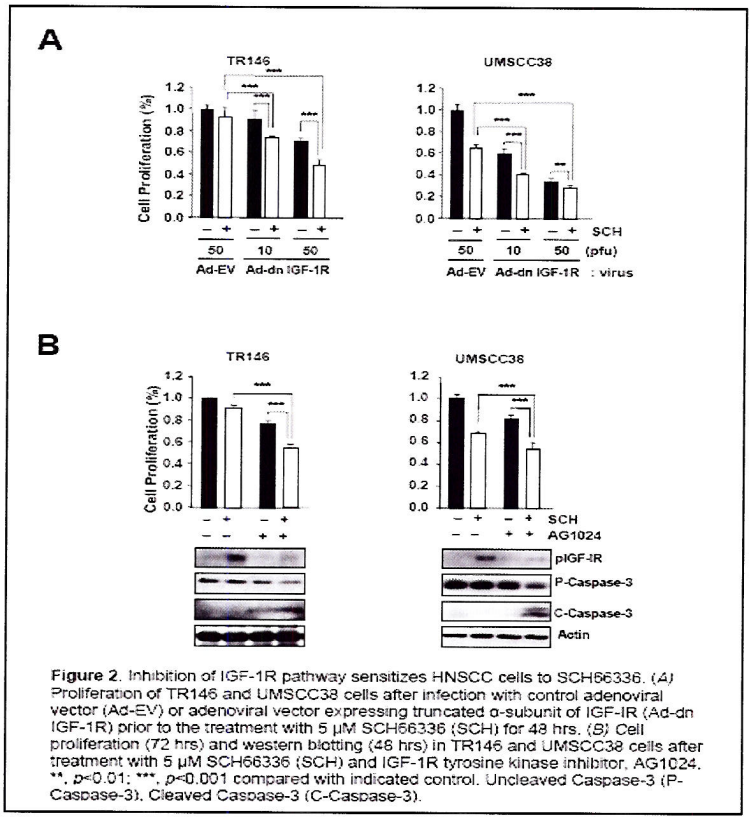


Figure 2. Inhibition of IGF-1R pathway sensitizes HNSCC cells to SCH66336. (*A*) Proliferation of TR146 and UMSCC38 cells after infection with control adenoviral vector (Ad-EV) or adenoviral vector expressing truncated α-subunit of IGF-IR (Ad-dn IGF-1R) prior to the treatment with 5 μM SCH66336 (SCH) for 48 hrs. (*B*) Cell proliferation (72 hrs) and western blotting (48 hrs) in TR146 and UMSCC38 cells after treatment with 5 μM SCH66336 (SCH) and IGF-1R tyrosine kinase inhibitor, AG1024. **, $p<0.01$; ***, $p<0.001$ compared with indicated control. Uncleaved Caspase-3 (P-Caspase-3). Cleaved Caspase-3 (C-Caspase-3).

The viability of a number of TR146 and UMSCC38 cells was significantly decreased by the SCH66336 treatment when IGF-1R pathway was inhibited. Moreover, combined treatment with SCH66336 and AG1024 induced marked decreases in the levels of pIGF-1R and concomitant cleavage of caspase-3.

Further, we investigated any correlation of the apoptotic effects of SCH66336 with the expression of apoptosis (IAP) family members, survivin and XIAP, by performing FACS (Figure 3A) and Western blot (Figure 3*B*) analyses in the HNSCC cell lines. TR146 showed a very mild increase in apoptotic cell population (Figure 3*A*) and active caspase-3 (Ac-Caspase-3) and XIAP expression with no detectable changes in survivin expression when SCH66336 was used (5 μM for 48 hrs) in 0.1 % FBS (Figure 3*A*). In the same condition, UMSCC38 and SqCC/Y1 cells showed increases in apoptotic cell population and expression of active caspase-3 and decreases in XIAP and survivin expression (Figure 3*A*). However, when SCH66336 was tested in the presence of 10%FBS, TR146, UMSCC22A, and UMSCC38 cells all showed dramatic increase in survivin expression with no change in pro- and active-caspase-3 levels. In contrast, SqCC/Y1, UMSCC10B, and SqCC35 cells showed a dramatic decrease in survivin and procaspase-3 levels in association with increases in active caspase-3 after the SCH66336 treatment (Figure 3*C*). XIAP expression remained unchanged in these cells after the drug treatment in the same condition. Consistent with the findings in HNSCC cell lines, survivin expression was markedly increased in H460 and 226Br NSCLC cell lines, which showed no significant difference in cell proliferation and pro- and active caspase-3 expression after the SCH66336 treatment (Figure 3*D*). Whereas, H226B cells showed significant decrease in cell number and survivin protein level in association with caspase-3 cleavage after the drug treatment (Figure 3*D*).

All these data suggested that expression of survivin, but not XIAP, may have been involved in the apoptotic activities of SCH66336 in HNSCC and NSCLC cells. Indeed, knock-down of survivin expression abolished resistance to SCH66336 and induced apoptosis in the TR146 and UMSCC 38 cells as determined by FACS (Figure 4A) and western blot analysis on survivin and caspase-3 (Figure 4B). Moreover, overexpression of survivin by the use of adenoviral vector protected SCH66336-sensitive HNSCC cells (SqCC/Y1 and UMSCC10B) from the apoptotic activities of SCH66336 (Figure 4B).

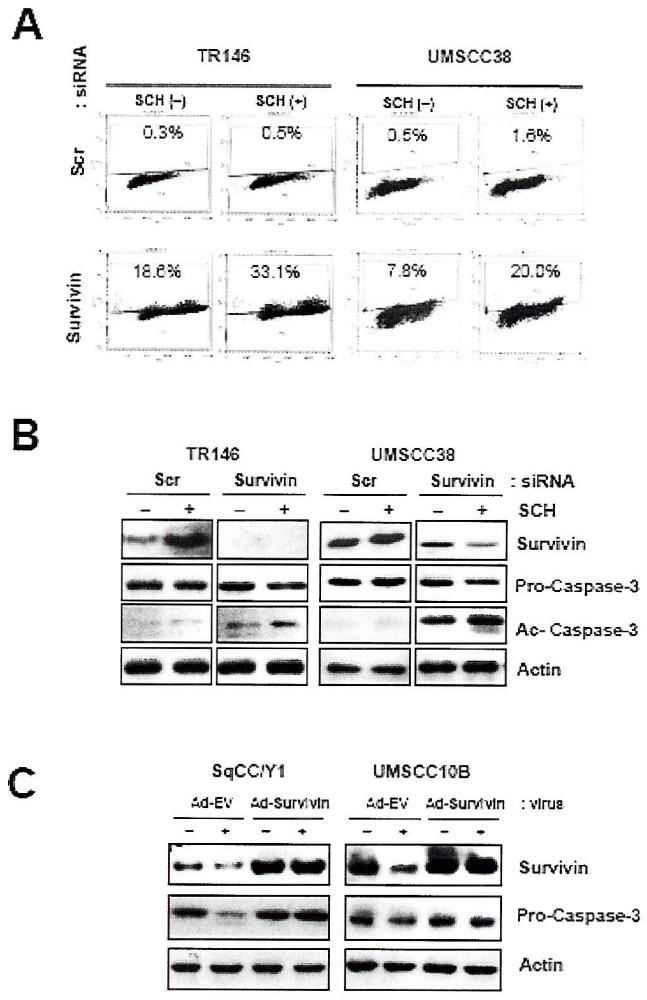


Figure 4. Survivin mediates resistance to SCH66336 in cancer cells. The indicated cells were transfected with control(Scr) or survivin siRNA or infected with control (Ad-EV) or survivin (Ad-Survivin) adenoviral vector. After the SCH66336 treatment (5 μM, 48 hrs), the cells were subjected to Flow cytometry-based, TUNEL assay (*A*) and/or Western blotting on survivin, pro- and active-caspase-3 and Actin (*B, C*) .

Thus, our results suggest that expression of pIGF-1R, pAkt, pmTOR, and survivin may serve as predictive markers for SCH66336 responsiveness in HNSCC.

Specific Aim 3.5 ***To study in vivo and in vitro effects of farnesyl transferase inhibitors and tyrosine kinase inhibitors in mouse models and, ultimately, in humans with lung cancer.***

(PI: Guillermina Lozano, Ph.D.)

Update

As briefly mentioned in Aim 3.1, we have now asked whether mice inheriting *K-ras* and *p53* mutations could serve as a model for preventive and therapeutic studies. While the initial studies were designed to treat $p53^{R172H\Delta g/+}$; $K\text{-}Ras^{LA1/+}$ mice with Iressa and FTI, a new anti-cancer drug, erlotinib (tarceva), has more clinical promise. Therefore, we used erlotinib in this new mouse model of lung cancer.

Eleven mice at approximately three months of age were treated once daily with a dose of 100 mg/kg/day. erlotinib was well tolerated by all mice. No significant side effects were observed. After treatment with erlotinib or vehicle for 30 days, mice were sacrificed and autopsied. Visible lesions on the lung surface were counted at the time of autopsy. The mean number of lesions on the lung surface was significantly reduced in mice given erlotinib (p=0.0072, Figure 5A). Chest imaging by micro-CT was also used to monitor tumor growth in 8 live mice before and after treatment. In these mice, all clearly visible lesions by micro-CT at the beginning of treatment were examined at the end of treatment. Treatment with erlotinib reduced the size or stabilized the growth of many but not all tumors (Figure 5B, C). Resistance to erlotinib suggested that these tumors acquired additional genetic changes. In the group given vehicle only, most lung tumors grew larger. Since erlotinib targets the epidermal growth factor receptor (EGFR), we examined adenocarcinomas for its expression. EGFR was expressed in all 15 lung adenocarcinomas examined and was present in both cytoplasmic and nuclear compartments (Figure 5D).

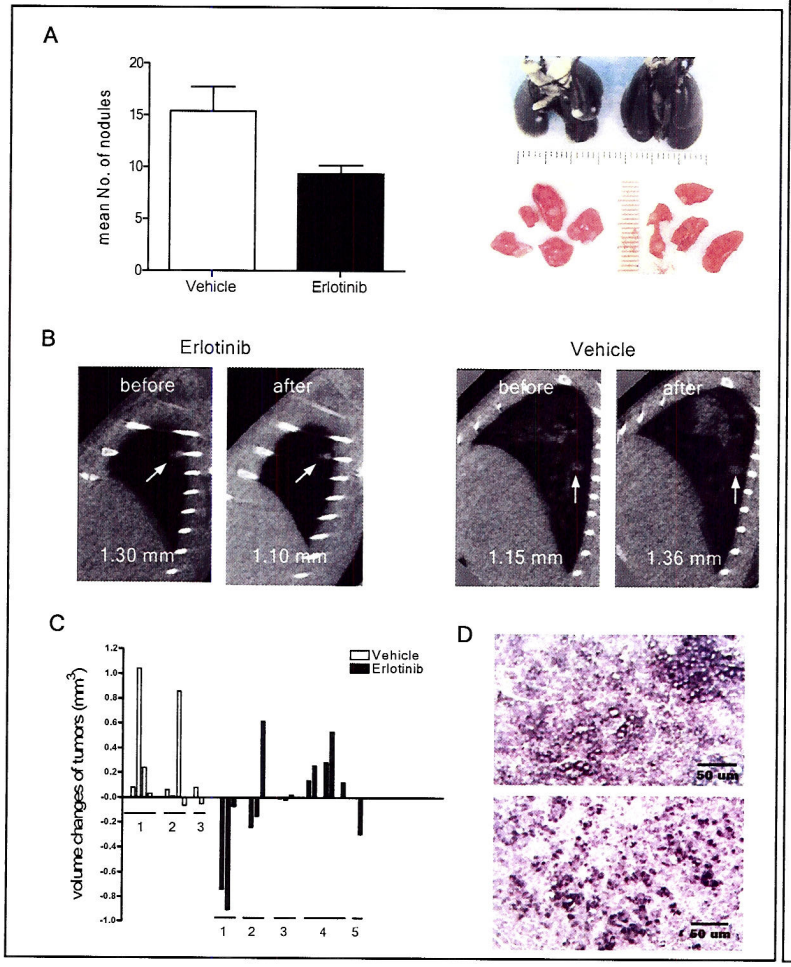


Figure 5. Erlotinib (Tarceva), a novel small molecule drug used to treat human lung adenocarcinoma, was tested in $p53^{R172H\Delta g/+}$ $K\text{-}ras^{LA1/+}$ mice. **A.** The mean number of lesions on the lung surface was greatly reduced in mice of Erlotinib group compared with that in the vehicle group (unpaired t test, p=0.0072). Lung lesions were counted at autopsy of mice with Erlotinib (n=11) or vehicle (n=5). Data were presented as mean values ± SEM. Photographs of lesions on the lung surface after treatment with vehicle (left) or Erlotinib (right), stained with India ink (top) or not (bottom). **B.** A representative lesion (arrows) was shown by micro-CT scans before and after treatment with Erlotinib or vehicle. Images show the largest measurement of the same lesion before and after treatment.Numbers in mm indicate the longest width of the lesion identified by an arrow. **C.** The volume changes of tumors during 30 days of treatment with vehicle (open bar) or Erlotinib (solid bar). Each bar represents the volume change of an individual lung tumor. The number below the bars represents the mouse number. **D.** The expression of EGFR in lung tumors of double mutant mice at 3-4 months of age by immunohistochemistry.

Thus, we show that erlotinib significantly reduces the mean number of lesions on the lung surface. Micro-CT monitoring demonstrates that erlotinib reduces or stabilizes the growth of many, but not all lung tumors, similar to clinical scenarios in lung cancer patients treated with erlotinib. A molecular analysis of resistant tumors may yield valuable insight into the changes that contribute to resistance.

The results were submitted for publication but more data is needed. We will thus seek additional funds from other sources to pursue these studies.

Specific Aim 3.6 ***To measure differences in gene expression between lung tumors that do or do not show metastasis, and in metastatic lesions themselves using the Affymetrix gene chip system.***

(PI: Guillermina Lozano, Ph.D.)

Update

As reported last year, we have collected samples and begun these studies. The quality of the mRNA was measured and the cRNA generated and hybridized to Affymetrix chips. We have evaluated the data with a bioinformatician. Analysis of the array data indicate that the primary tumors and metastases segregate separately. A total of 79 genes were differentially expressed between the data sets with a p value less than 0.001 (Figure 6). We verified by the Western blot analysis that several of the genes in tumors, e.g., vimentin and Bub1b, were overexpressed and that C-cam1 was down modulated in metastases.

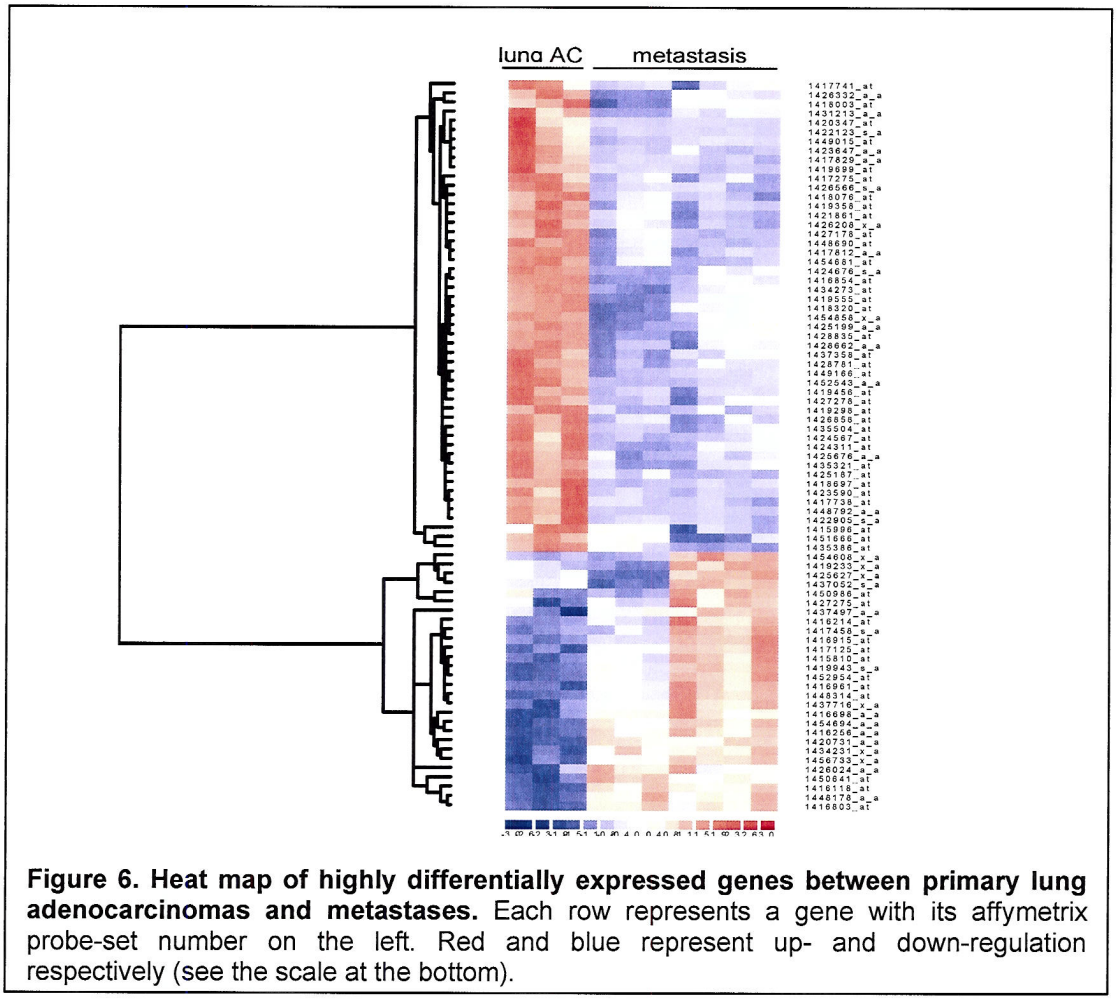


Figure 6. Heat map of highly differentially expressed genes between primary lung adenocarcinomas and metastases. Each row represents a gene with its affymetrix probe-set number on the left. Red and blue represent up- and down-regulation respectively (see the scale at the bottom).

Specific Aim 3.7 ***To perform array CGH experiments to determine if other genomic changes have occurred.***

Specific Aim 3.8 ***To perform LOH studies at specific loci (if warranted from the data obtained in Specific Aim 3.7).***

(Leader: Guillermina Lozano, Ph.D.)

These studies will not be further pursued here due to lack of funds. Additional sources of the funds will be sought to continue the studies.

Specific Aim 3.9 ***To evaluate GFE-1 peptide effects on blocking lung metastases in a rat model.***

(PI: Yun W Oh, M.D)

This Aim was concluded in 2005; Dr. Yun Oh discontinued participation in the BESCT program as noted in an official letter to Dr. Julie Wilberding.

Key Research Accomplishments:

- Developed the Phase 1b combination trial of lonafarnib with docetaxel in biopsy-accessible solid tumor patients based on the findings of Aim 3.2 (supported by NIH/NCI P01, Sanofi-Aventis pharmaceuticals, and Schering Plough). The trial was activated and has accrued 12 patients.
- M. D. Anderson Institutional Compliance Office reviewed the protocol of the Phase I trial of DOTAP:cholesterol-FUS1 liposome complex and decided to transfer the manufacture of the liposome complex from the facility at Baylor College of Medicine to M. D. Anderson Cancer Center's GMP facility where the study drug was successfully produced, and the trial was reopened. To date, 13 patients have been entered on the study, but a maximum tolerated dose (MTD) has not been reached. Enrollment to the trial continues.
- Determined that FTI SCH66336 induced IGF-1R/Akt/mTOR-dependent survivin expression, which in turn protected HNSCC and NSCLC cells from the apoptotic activities of SCH66336.
- Determined that erlotinib significantly reduced the mean number of lesions on the lung surface of mice inheriting *K-ras* and *p53* mutations in which erlotinib reduced or stabilized the growth of many, but not all lung tumors, similar to clinical scenarios in lung cancer patients treated with erlotinib.
- Found that a total of 79 genes were differentially expressed between the primary and metastases tumors, and verified that vimentin and Bub1b were overexpressed and C-cam1 was down modulated in metastases.

Reportable Outcomes:

Publications in peer-reviewed journals

- Morgillo F, Lee H-Y. Lonafarnib in Cancer Therapy: A Review. *Expert Opin Investig Drugs* 15(6):709-19, 2006.

Manuscripts in Preparation, review, or revision

- Oh SH, Jin Q, Hong WK, Lee HY. Insulin-like Growth Factor Receptor Signaling Pathways Induces Resistance to Apoptotic Activities of SCH66336 (Lonafarnib) through Akt/mTOR-mediated Increases in the Survivin Expression. (in preparation.)
- Zheng S, El-Naggar AK, Kim ES, Kurie JM, Lozano G. A Genetic Mouse Model for Metastatic Lung Cancer with Gender Differences in Survival. *Oncogene*, 2007 (In review).

Conclusions:
The original tasks (Aims) of Project 3 have been completed, but its extended tasks (Aims 3.5-3.8) are still ongoing. At this point, we conclude that 1) we have established a faithful mouse model that recapitulates the metastatic nature of human lung cancer that will be invaluable to further probe the molecular basis of metastatic lung cancer and for translational studies; 2) We have demonstrated the synergistic effect of lonafarnib (SCH66336) with taxanes in anti-proliferation activity; and 3) our findings suggest that NSCLC and HNSCC cells develop FTI SCH66336 resistance by inducing survivin expression through an IGF-1R/Akt-dependent pathway. Thus, combining inhibitors of IGF-1R, Akt, or survivin with FTI SCH66336 may be an effective anticancer therapeutic strategy for patients with HNSCC or NSCLC.

Developmental Research Project: A Genetic/Combinatorial Algorithmic Strategy for Anticancer Therapy Development

(PI: Ralph Zinner, M.D)

Targeted therapeutic agents are highly promising in combination because they are both well-tolerated and interact with the targets that cause cancer. However, with new drugs added to the list, the number of possible combinations rises exponentially beyond the capacity of any foreseeable technology to fully screen. In addition, molecular insight often fails to predict clinical performance of single agents, a difficulty that will likely remain as these drugs are combined. We thus propose a direct functional screen of combinations as a complement to the molecular insight-based approach, MACS (Medicinal Algorithmic Combinatorial Screen), to identify promising combinations that would be otherwise impossible to be found through a simple screen alone. The foundation of MACS is a genetic algorithm. The study adopts a preclinical screen that assesses anticancer efficacies of combinations with cell proliferation assays.

Specific Aim 1: ***To determine feasibility of screening process (robots, cell death assays, combining drugs).***

This will be pursued later once we have access to a robot facility.

Specific Aim 2: ***To determine the range of outcomes and patterns of cellular response from an initial screening of drug combinations.***

One of the requirements for MACS will be the presence of some degree of linearity or higher order relationships among combinations. This means that knowing the behavior of some combinations from a set of drugs enables prediction of the behavior of other combinations from the same drugs. If there were perfect linearity, knowing the behavior of all the single agents would allow prediction of the behavior of all large drug combinations that are formed from them. At the opposite extreme, if there is no linearity, it would not be possible to improve from one generation to the next, a requirement of MACS. At present, little is known of the degree of relatedness between the large combinations of anticancer agents.

Update
As reported last year, initial analyses of several power sets of 6 drugs in A549 cells showed that 67% of the variance could be explained by linear combinations. We have conducted 4 MAC screens (2 since the last report).

More than 500 combinations were studied through the process and will be analyzed for linearity. In addition, we will extend Aim 2 by conducting data mining and using pattern recognition analytical tools to make predictions about novel combinations. We are exploring a complementary method to

MACS that will mine many combinations studied using powerful analytical tools to learn whether we can make predictions about combinations not yet studied. If such strategy is valid, insights derived from old or ongoing MACS assays could influence the design of algorithms that guide the MAC screens. This goal may be achievable through the recent establishment of the collaboration with Dr. Elena Popova of Operations Research and Industrial Engineering at The University of Texas at Austin, and Dr. Paul Damien at the McCombs School of Business, The University of Texas at Austin.

Specific Aim 3: ***To develop genetic algorithm to guide selection and identification of promising combinations of drugs.***

We screened combinations from 19 drugs (Table 1) (524,288 possible cocktails) for inhibition of the lung cancer cell line, A549, guided by MACS. We applied a hybrid nonlinear optimization algorithm (MACS) to search for effective cocktails composed of up to 19 different drugs each with a fixed single agent IC_{10} dose. The search revealed a cocktail that performed 4.18 standard deviations above the mean of a random set of cocktails after assaying only 370 combinations.

Table 1. List of 19 drugs used in the MACS assays.

Name	*IC 10 uM*	1x dose	2x dose	4x dose
indirubin	1	1.17	.95	1.01
Anisomycin	0.015	.97	.82	.69
LY294002	2	.99	.88	.92
SP 600125	5	.85	.60	.46
Deguelin	12	.63	.57	.43
Rapamycin	6	.73	.64	.57
cisplatin	5	.89	.71	.57
imatinib mesylate	3	1.11	.94	1.05
gemcitabine	0.004	.89	.97	.72
Bortezomib/Velcade	0.005	.82	.47	.44
(Decitabine)	2	.99	.95	1.00
PD 168393	5	.99	.76	.59
4-HPR (retinamide)	5	.93	.67	.34
ATRA (Tretinoin)	15	.84	.76	.64
CD437	0.3	.89	.55	.40
MX3350-1	0.5	1.06	.88	.72
ST1926	0.05	1.08	.95	.72
SAHA	2	.78	.56	.34
SCH66336	5	.72	.75	.56

The results from the first pair of MAC screens are shown in Figure 1. In Figure 1, fitness is indicated on the y-axis, the higher up the more fit. The x-axis indicates the generation. Each red dot indicates the fitness of a combination. The black dots do not indicate the results from the generation. Rather, they represent the fitness values of selected combinations from prior generations that were used to help calculate/select combinations to be altered for the subsequent combination. Thus, for the purposes of understanding increasing fitness/enrichment of the fitness of the pool of combinations across the generations, the red dots are informative.

Since the last report, we did a multiple comparison analysis of pilot A-Experiment 0 that showed improvement in fitness across the generations (Figure 2). This formally indicates a nonrandom enrichment for fitness of the combinations through MACS. The fittest combination of drugs identified was SAHA, bortezomib, and 4HPR (4SB) after the local search was conducted around the combinations in the experiment 0. Experiment 1 as can be seen by inspection did not have the same level of enrichment. Therefore, we opted to further explore the results from experiment 0 alone both in terms of the statistical analyses and isolation of a combination of interest (4SB).

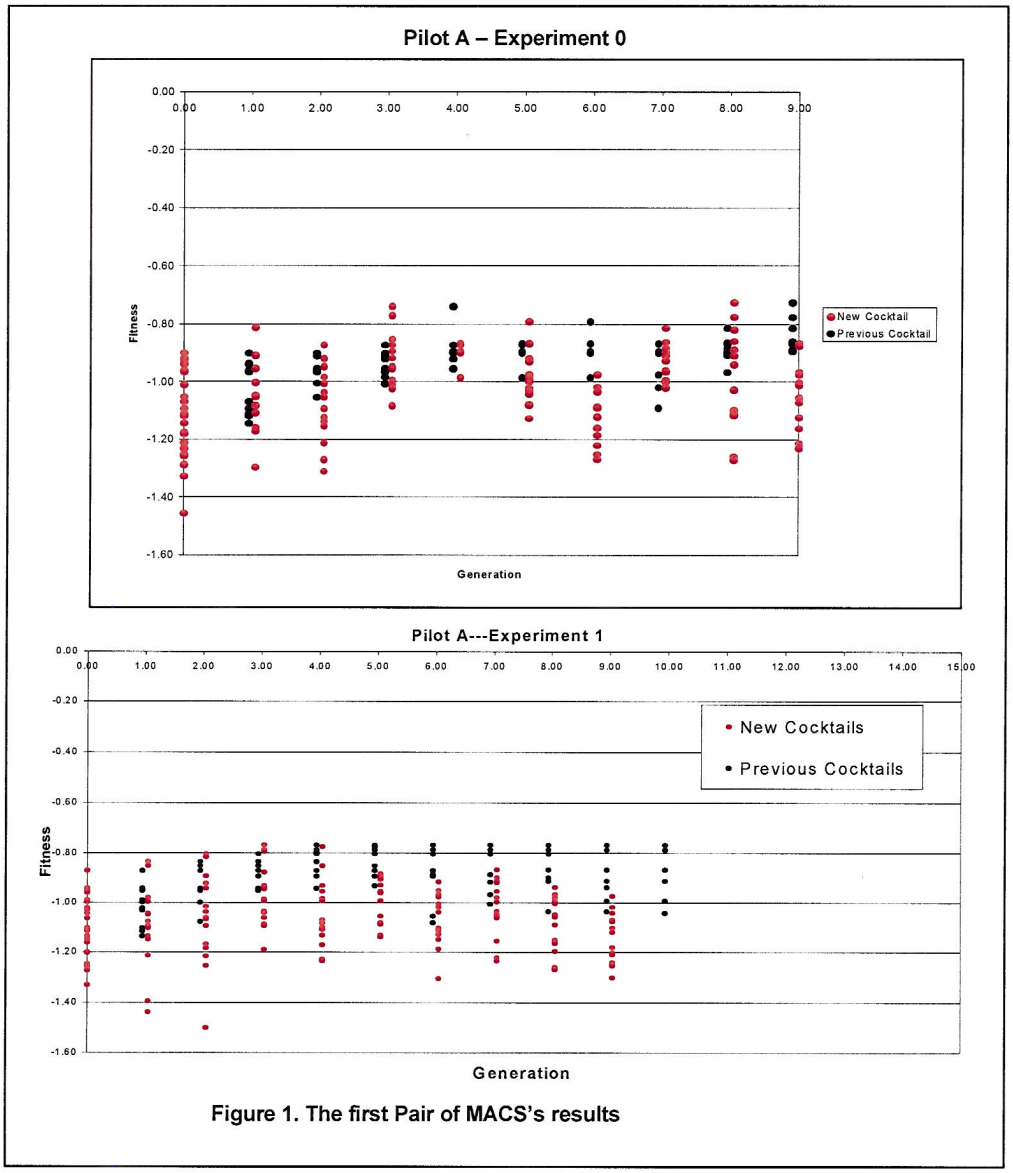


Figure 1. The first Pair of MACS's results

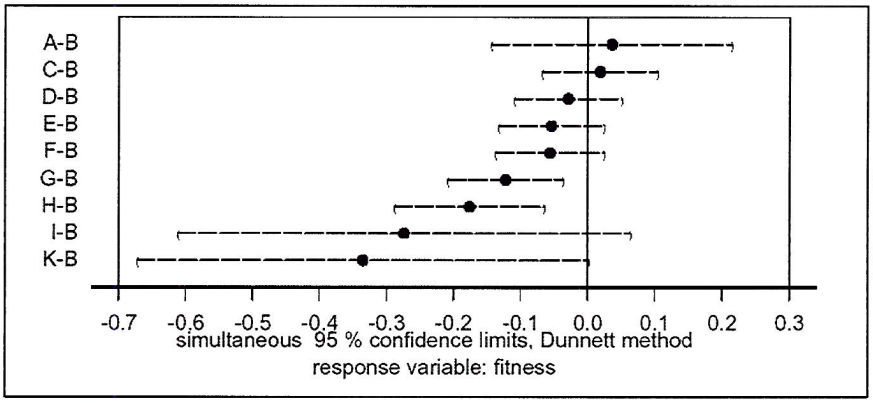


Figure 2. A multiple comparison analysis of pilot A experiment 0 in fitness across the generations.

From the second pair of MAC screens, we have conducted 2 additional searches (the Hill Climb and the modified version of the genetic algorithm). The Hill Climb was able to identify the same combination, 4SB (4HPR, SAHA, and bortezomib) in a screen of only 70 combinations over 4

generations. The entire experiment took only 2 weeks indicating the efficiency with which the MACS can be done (Figure 3A and B).

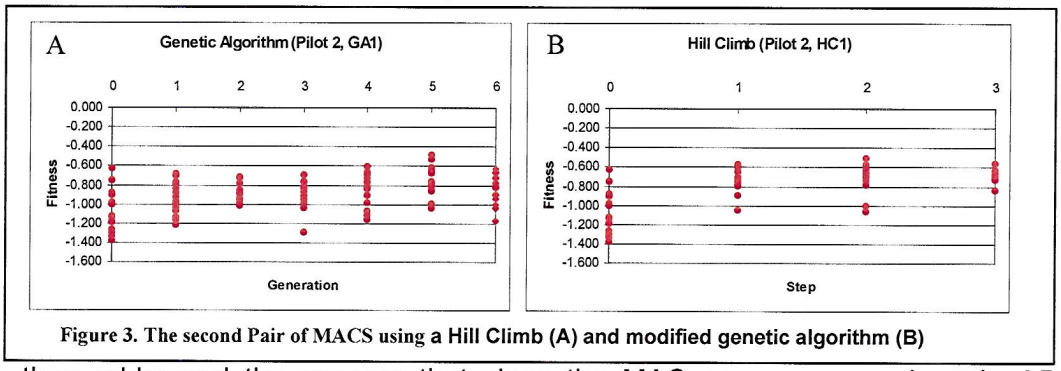
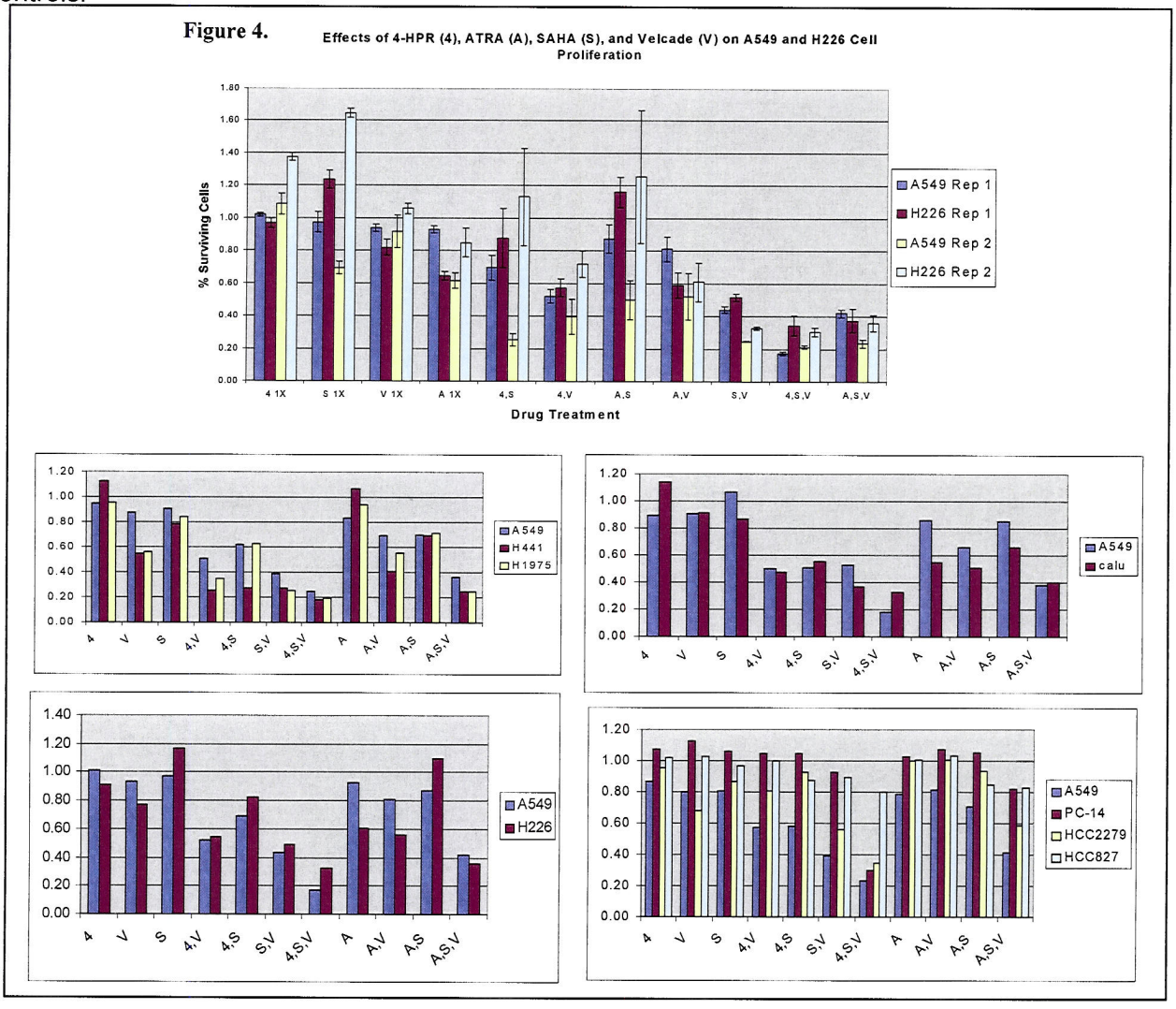


Figure 3. The second Pair of MACS using a Hill Climb (A) and modified genetic algorithm (B)

We then addressed the concern that since the MAC screens were done in A549 alone, any combinations identified would have limited relevance. We therefore looked at other 9 NSCLC cell lines by testing all the powersets (three pairs and all single agents-4SB, 4S, 4B, SB, 4, S, B, no treatment control). We found that the triplet combination (4SB) reliably inhibited cells irrespective of cell lines (the doses were unchanged from the IC_{10} of A549) (Figure 4). In Figure 4, each of the graphs below represents a different experiment containing at least one novel cell line. For all experiments, all the powersets of 4SB, 4S, 4B, SB, 4, S, B, no treatment control were tested in A549. This served as a control in addition to the usual single agent 1x, 2x and 4x single agent controls.

Figure 4. Effects of 4-HPR (4), ATRA (A), SAHA (S), and Velcade (V) on A549 and H226 Cell Proliferation



Since this was not the case for the single agents or all the pairs, it suggests that more drugs may indeed have a higher probability of being effective therapy given the known variety of clones even within the same tumor. In addition, we substituted ATRA for 4HPR, both retinoids. Though ATRA was also effective, it was less so in the 9 cell lines. This indicates that molecular insight though predictive, is somewhat limited. In turn, this conclusion supports the premise that a functional screen that composes the MACS can serve as a useful complement to molecular insight based therapy development.

We also did an assessment of synergy using the CalcuSyn (Biosoft Ltd. Cambridge, United Kingdom). It demonstrated increasing synergy from doublet combinations (2 of them) to the triplet combinations (3 of them) (Table 2).

A549

Drug	CI Values (A549)		
	ED50	ED75	ED90
4S (2.5:1)	0.99328	1.04689	1.10725
4B (1000:1)	0.86567	0.88395	0.90464
SB (400:1)	0.6712	0.53133	0.42067
4SB(1000:400:1)	0.8783	0.72439	0.59901

H226

Drug	CI Values (H226)		
	ED50	ED75	ED90
4S (2.5:1)			
4B (1000:1)	0.81697	0.56337	0.4027
SB (400:1)	0.608	0.49556	0.40391
4SB(1000:400:1)	0.77534	0.52337	0.3662

Table 2. The 3-triplet was compared to other members of its own powerset in both A529 and H226. Where values are less than 1, there is synergy.

Although we showed that an automatic search can efficiently identify novel combinations without using molecular insight, MACS could readily integrate such insights into the search itself; for example, by giving a greater fitness value to combinations that contain certain smaller combinations. We are presently analyzing lysates derived from A549 and H226 cells treated by the powerset 4SB. They will be assessed using a reverse antibody array in collaboration with Dr. Gordon Mills. More than 50 signal transduction pathways will be checked. This is not only of interest as a potential guide to important signaling nodes within a web of interacting messengers, but also as an opportunity to study how to integrate such insights into MACS.

In addition, we will shortly do an intensive analysis of patterns across the many hundreds of combinations assayed thus far to assess the potential of such methods to predict novel combinations. In addition to the potential utility of any given combination found this way, the method itself may be integrated into MACS as well.

We have prepared a manuscript summarizing detailed findings of the study and submitted it recently to *Molecular Cancer Therapeutics* (Zinner et al., 2007).

Key Research Accomplishments:

- Demonstrated that MACS does indeed enrich for sets of more fit (fitness is a function of increased inhibition and fewer drugs) combinations independent of molecular insight (validated statistically).
- Identified a triplet combination (4SB) that is synergistic in 2 cell lines evaluated and at least additive in a total of 10 NSCLC cell lines.

Reportable Outcomes:

Manuscript in preparation

- Zinner RG, Barrett BL, Popova E, Damien P, Volgin AY, Gelovani JG, Lotan R, Pisano C, Lippman SM, Mills GB, Mao L, Miller JH. Medicinal Algorithmic Combinatorial Screen (MACS) Is a Novel, Rapid, and Efficient Approach to the Identification of Therapeutic Combination Drug Regimens. *Mol Cancer Therap*, 2007 (submitted).

Grants

Based on the data generated from the project, we have been awarded two seed grants, and are preparing two additional grants:

Funded:

- IRG (Institutional Research Grant): 9/30/06 - 6/30/07.
  This funding is to characterize the toxicity and efficacy of the triplet we identified through MACS, 4SB (4HPR plus SAHA plus bortezomib) in a nude mouse model. In addition, we are acquiring tumor and mouse host tissues to do molecular-pathological analyses. Principal Investigator: Ralph Zinner, M.D.
- UT Biomedical Engineering Seed Grant: 1/15/2007- 12/14/2007.
  Data Mining Combinations Derived from the Medicinal Algorithmic Combinatorial Screen (MACS). Through this grant, we will be using data from more than 500 cocktails screened through the MACS to analyze patterns of efficacy and the composition of the combinations. Baysean techniques will be used to generate novel candidate combinations which will then be tested using clonigenic *in vitro* assays.

In preparation:

- NCI R01, May 2007. We are proposing to analyze a reverse antibody array data of the triplet, 4SB, across multiple NSCLC cell lines (some data are already generated), and then incorporate molecular insights into a MACS.
- NCI R21; To further develop the triplet, 4SB, in a head and neck mouse model developed by Dr. Reuben Lotan at UT M. D. Anderson Cancer Center.

Presentations

- MACS, Medicinal Algorithmic Combinatorial Screen. Ralph Zinner. Keystone Symposia: Molecular Targets for Cancer Prevention. Lake Tahoe, CA, March 9, 2006.

Conclusions:

This study demonstrates the potential feasibility for screening drug combinations of arbitrary size using the Medicinal Algorithmic Combinatorial Screening (MACS) method, which can efficiently identify highly-fit combinations of anticancer agents without prior molecular or functional insight into the interactions of the combined drugs.

KEY RESEARCH ACCOMPLISHMENTS

Project 1: Study Mechanisms of Molecular Alterations in Lung Cancer

- Demonstrated that HDGF involves anchorage-independent growth, invasion, and neovasculature formation of NSCLC cells during lung cancer progression.
- Demonstrated HDGF as a novel therapeutic target for lung and other major cancer types.
- Developed monoclonal antibodies against HDGF as potential therapeutic agents and demonstrate their therapeutic efficacies in treating lung and pancreatic cancers in tumor xenograft models.
- Successfully licensed the HDGF technology to a major pharmaceutical company for further development towards potential clinical applications.
- Discovered an important role of ΔDNMT3B variants in differential regulation of promoter DNA methylation, which may lead to development of novel cancer therapeutic strategies.

Project 2: Develop Novel Strategies for Lung Cancer Chemoprevention

- Completed revisions of the protocol for the Phase I combination trial of bexarotene and celecoxib in solid tumors. Received approval from the Emory IRB on January 5, 2007, and awaiting final approval from the DoD.
- Found that the sensitivity to the Cox-2 or 5-Lox inhibitors was not directly associated with the expression levels of COX-2, 5-LOX, or FLAP, but was correlated with the production of the corresponding metabolites.
- Demonstrated that 4HPR exerts a borderline significant suppression of the development of lung adenocarcinomas in NNK-exposed Gprc5a knockout mice. The anti-angiogenic and growth inhibitory plus pro-apoptotic effects of 4HPR could account for its chemopreventive activity.

Project 3: Implement Experimental Molecular Therapeutic Approaches for Lung Cancer

- Developed the Phase 1b combination trial of lonafarnib with docetaxel in biopsy-accessible solid tumor patients based on the findings of Aim 3.2 (supported by NIH/NCI P01, Sanofi-Aventis pharmaceuticals, and Schering Plough). The trial was activated and has 12 patients accrued.
- M. D. Anderson Institutional Compliance Office reviewed the protocol of the Phase I trial of DOTAP:cholesterol-FUS1 liposome complex and decided to transfer the manufacture of the liposome complex from the facility at Baylor College of Medicine to M. D. Anderson Cancer Center's GMP facility where the study drug was successfully produced, and the trial was reopened. To date, 13 patients have been entered on the study, but a maximum tolerated dose (MTD) has not been reached. Enrollment to the trial continues.
- Determined that FTI SCH66336 induced IGF-1R/Akt/mTOR-dependent survivin expression, which in turn protected HNSCC and NSCLC cells from the apoptotic activities of the SCH66336.
- Determined that erlotinib significantly reduced the mean number of lesions on the lung surface of mice inheriting *K-ras* and *p53* mutations in which erlotinib reduced or stabilized the growth of many, but not all lung tumors, similar to clinical scenarios in lung cancer patients treated with erlotinib.
- Found that a total of 79 genes were differentially expressed between the primary and metastases tumors, and verified that vimentin and Bub1b were overexpressed and C-cam1 was down modulated in metastases.

Developmental Research Project: A Genetic/Combinatorial Algorithmic Strategy for Anticancer Therapy Development

- Demonstrated that MACS does indeed enrich for sets of more fit (fitness is a function of increased inhibition and fewer drugs) combinations independent of molecular insight (validated statistically).
- Identified a triplet combination (4SB) that is synergistic in 2 cell lines evaluated and at least additive in a total of 10 NSCLC cell lines.

REPORTABLE OUTCOMES

Manuscripts published in peer-reviewed Journals

- Morgillo F, Lee H-Y. Lonafarnib in Cancer Therapy: A Review. *Expert Opin Investig Drugs* 15(6):709-19, 2006.
- Schroeder CP, Kadara H, Lotan D, Woo JK, Lee HY, Hong WK, Lotan R. Involvement of Mitochondria and Akt-Signaling Pathway in Augmented Apoptosis Induced by a Combination of Low Doses of Celecoxib and 4HPR in Premalignant Human Bronchial Epithelial Cells. *Cancer Res,* 66: 9762-70, 2006.
- Schroeder CP, Yang P, Newman RA, Lotan R. Simultaneous inhibition of COX-2 and 5-LOX activities augments growth arrest and death of premalignant and malignant human lung cell lines. *J. Exptl. Therap. Oncol* (in press), 2007.
- Wang J, Walsh G, Liu D, Lee JJ, Mao L. Expression of *ΔDNMT3B* Variants and its association with promoter methylation of *p16* and *RASSF1A* in primary non-small cell lung cancer. *Cancer Res*, 66:8361-8366, 2006.
- Wang L, Wang J, Sun SY, Rodriguez M, Yue P, Jang SJ, Mao L. A Novel DNMT3B subfamily, ΔDNMT3B, is the predominant form of DNMT3B in non-small cell lung cancer. *Internatl J Oncol*, 29:201-207, 2006.
- Zhang J, Ren H, Yuan P, Lang W, Zhang L, Mao L. Down-regulation of hepatoma-derived growth factor inhibits anchorage-independent growth and invasion of non-small cell lung cancer cells. *Cancer Res* (Priority report) 66:18-23, 2006.

Manuscripts in preparation, review, or revision

- Oh SH, Jin Q, Hong WK, Lee HY. Insulin-like Growth Factor Receptor Signaling Pathways Induces Resistance to Apoptotic Activities of SCH66336 (Lonafarnib) through Akt/mTOR-mediated Increases in the Survivin Expression. (In preparation)
- Wang L, Jin W, Ren H, Cote GJ, Mao L. Polypyrimidine Tract-binding Protein (PTB) is involved in Altering *CEACAM1* Splicing Pattern in Lung Cancer Cells. 2007. (In preparation).
- Zheng S, El-Naggar AK, Kim ES, Kurie JM, Lozano G. A Genetic Mouse Model for Metastatic Lung Cancer with Gender Differences in Survival. *Oncogene*, 2007 (in review).
- Zinner RG, Barrett BL, Popova E, Damien P, Volgin AY, Gelovani JG, Lotan R, Pisano C, Lippman SM, Mills GB, Mao L, Miller JH. Medicinal Algorithmic Combinatorial Screen (MACS) Is a Novel, Rapid, and Efficient Approach to the Identification of Therapeutic Combination Drug Regimens. *Mol Cancer Therap*, 2007 (submitted).

Abstracts

- Mao L, Ren H, Chu Z, Yuan P. Therapeutic activity of neutralizing monoclonal antibodies targeting hepatomaderived growth factor in cancer xenograft models. The *ASCO Annual Meeting 42:2517*, 2006.

- Zhang J, Mao L. SiRNA targeting hepatoma-derived growth factor (HDGF) inhibits growth of non-small cell lung cancer in xenograft models. The 97th *AACR annual meeting, Abstract#:* 5135, 2006.

Presentations

- MACS, Medicinal Algorithmic Combinatorial Screen. Ralph Zinner. Keystone Symposia: Molecular Targets for Cancer Prevention. Lake Tahoe, CA, March 9, 2006.

Grants

- IRG (Institutional Research Grant): 9/30/06 - 06/30/07.
  This funding is to characterize the toxicity and efficacy of the triplet we identified through MACS, 4SB (4HPR plus SAHA plus bortezomib) in a nude mouse model. In addition, we are acquiring tumor and mouse host tissues to do molecular-pathological analyses. Principal Investigator: Ralph Zinner, M.D.

- UT Biomedical Engineering Seed Grant: 01/15/07- 12/14/07.
  Data Mining Combinations Derived from the Medicinal Algorithmic Combinatorial Screen (MACS). Through this grant, we will be using data from more than 500 cocktails screened through the MACS to analyze patterns of efficacy and the composition of the combinations. Baysean techniques will be used to generate novel candidate combinations which will then be tested using clonigenic *in vitro* assays.
  Investigator: Ralph Zinner, M.D.

Patents

- Mao L et al. Patent application No. 11611554 and International Patent Application Number: PCT/US2006/062176; Anti-Hyperproliferative Therapies Targeting HDGF. Filed December 15, 2006.
- Mao L et al. Method of treating a cancer. Patent application No. 20060115829.

CONCLUSIONS

In the 6th year grant period, Project 1 has been successfully completed. The clinical trial of Project 2 (Aim 2.1) will be opened soon, and the tasks (Aim 2.1-2.2, 2.4) of Project 2 are still ongoing and expected to complete next year. The original tasks (Aims) of Project 3 have also been completed; its extended tasks (Aims 3.5-3.8) are still ongoing.

This year, we have 6 publications including 3 in *Cancer Research*, 1 in *International Journal of Oncology*, 1 in *Journal of Experimental Therapeutics of Oncology*, and 1 review article in *Expert Opinions of Investigational Drugs*, 2 meeting abstracts, and 4 manuscripts in preparation or submitted for review. Two patents were filed.

Project 1: We have demonstrated the importance of ΔDNMT3B, a novel subfamily of DNMT3B discovered in the project, in lung tumorigenesis. More importantly, we have shown that ΔDNMT3B variants play an important role in regulation of promoter methylation in a gene-specific manner. This discovery has potentially significant implications in the development of novel cancer therapies by regulating gene expression through modulating levels of individual ΔDNMT3B variants. These studies highlight the critical role of alternative splicing of pre-mRNA in lung tumorigenesis.

The discovery of HDGF as a key factor in lung cancer progression provides a potential biomarker to predict patients' outcomes and a potential target for treating lung cancer. A major success of the

project is the development of candidate therapeutic monoclonal antibodies. We have demonstrated that these antibodies are effective in inhibiting lung cancer growth *in vivo* through novel mechanisms. We have reached a major milestone by licensing the technology to a major biopharmaceutical company for antibody humanization and clinical development, a critical step to translate our laboratory research discoveries to patient care.

Proposed aims have been completed and the study is closed.

Project 2: Our data suggest that the combination of celecoxib and 4HPR or combinations of celecoxib and LOX inhibitors may be useful for lung cancer chemoprevention and treatment.

Project 3: The original tasks (Aims) of Project 3 have been completed, but its extended tasks (Aims 3.5-3.8) are still ongoing. At this point, we conclude that: 1) We have established a faithful mouse model that recapitulates the metastatic nature of human lung cancer that will be invaluable to further probe the molecular basis of metastatic lung cancer and for translational studies; 2) We have demonstrated the synergistic effect of lonafarnib (SCH66336) with taxanes in anti-proliferation activity; and 3) Our findings suggest that NSCLC and HNSCC cells develop FTI SCH66336 resistance by inducing survivin expression through an IGF-1R/Akt-dependent pathway. Thus, combining inhibitors of IGF-1R, Akt, or survivin with FTI SCH66336 may be an effective anticancer therapeutic strategy for the patients with HNSCC or NSCLC.

DRP: The study demonstrates the potential feasibility for screening drug combinations of arbitrary size using the Medicinal Algorithmic Combinatorial Screening (MACS) method, which can efficiently identify highly-fit combinations of anticancer agents without prior molecular or functional insight into the interactions of the combined drugs.

REFERENCES

- Chun KH, Lee HY, Hassan K, Khuri FR, Hong WK, Lotan R. Implication of protein kinase B/Akt and Bcl-2/Bcl-xl suppression by the farnesyl transferase inhibitor SCH66336 in apoptosis induction in squamous carcinoma cells. *Cancer Res* 63: 4796–800, 2003.
- Han JY, Oh SH, Morgillo F, Myers JN, Kim E, Hong WK, Lee HY. Hypoxia-inducible factor 1alpha and antiangiogenic activity of farnesyltransferase inhibitor SCH66336 in human aerodigestive tract cancer. *J Natl Cancer Inst* 97: 1272-86, 2005.
- Lee HY, Oh SH, Suh YA, Baek JH, Papadimitrakopoulou V, Huang S, Hong WK. Response of non-small cell lung cancer cells to the inhibitors of phosphatidylinositol 3-kinase/Akt- and MAPK kinase 4/c-Jun NH2-terminal kinase pathways: an effective therapeutic strategy for lung cancer. *Clin Cancer Res* 11: 6065-74, 2005.
- Mao L et al. Patent application No. 20060115829. Method of Treating a Cancer. Filed July 26, 2005.
- Marcus AI, Zhou J , Hamel E, Nivens M, El-Naggar A, Khuri FR, Giannakakou P. Novel target for the farnesyl transferase inhibitor, lonafarnib: increased microtubule acetylation underlies synergy with taxanes. *Cancer Res* 65:3883-93, 2005.
- Sun SY, Schroeder CP, Yue P, Lotan D, Hong WK, Lotan R. Enhanced Growth Inhibition and Apoptosis Induction in NSCLC Cell Lines by Combination of Celecoxib and 4HPR at Clinically Relevant Concentrations. *Cancer Biol & Ther* 4:407-13, 2005.
- Tang XM, Wu WG, Sun SY, Wistuba II, Hong WK, Mao L. Hypermethylation of the Death-Associated Protein-Kinase Promoter Attenuates the Sensitivity to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis in Human Non-Small-Cell Lung Cancer Cells. *Mol Cancer Res* 2:685-91, 2004.
- Wang J, Lee JJ, Wang L, Liu D, Lu C, Fan YH, Hong WK, Mao L. Value of *p16*^{INK4a} and *RASSF1A* promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. *Clin Cancer Res* 10:6119-25, 2004.

APPENDICES

A - C

APPENDIX A

Publications

Expert Opinion

Lonafarnib in cancer therapy

Floriana Morgillo & Ho-Young Lee†
†*M. D. Anderson Cancer Center, Department of Thoracic/Head & Neck Medical Oncology, Houston, TX, USA*

Farnesyl transferase inhibitors (FTIs) are anticancer agents that were designed to block the post-translational attachment of the prenyl moiety to C-terminal cysteine residue of Ras and thus inactivate it. Because Ras plays an important role in tumour progression and the *ras* mutation is one of the most frequent aberrations in cancer, FTIs have been expected to exert excellent therapeutic activities. Phase I and II clinical trials confirmed relevant antitumour activity and low toxicity; however, no improvement in overall survival has been reported in Phase III trials. The exact mechanism of action of this class of agents is currently unknown. Increasing lines of evidence indicate that the cytotoxic actions of FTIs are not due to the inhibition of Ras proteins exclusively, but to the modulation of other targets, including RhoB, the centromere-binding proteins and other proteins that have not yet been identified. This review describes the pharmacological and clinical data as well as mechanisms of action of FTIs, especially lonafarnib (SCH-66336), a non-peptidomimetic inhibitor that has shown anticancer activity.

Keywords: farnesyl transferase inhibitor, lonafarnib, Ras, SCH-66336, targeted therapy

Expert Opin. Investig. Drugs (2006) 15(6):709-719

1. Introduction

Recent advances in the understanding of cell biology and cancer genetics have permitted identification of novel therapeutic targets and led to the development of rational mechanism-based drugs. Farnesyl transferase inhibitors (FTIs) are among the first and most-studied oncogene-targeted agents. They were designed to prevent the function of Ras proteins by blocking the post-translational attachment of the prenyl moiety to its C-terminal cysteine, thereby inhibiting its membrane localisation and function (**Figure 1**). Protein prenylation is a post-translational modification in which either a farnesyl or geranylgeranyl isoprenoid is linked to specific cysteine residues of proteins via a thioether bond. These proteins belong to a group termed 'CAAX proteins', which is defined by a specific C-terminal motif that directs their modification. The CAAX group of prenylated proteins includes Ras and a multitude of guanosine triphosphate (GTP)-binding proteins and several protein kinases and phosphatases.

Ras proteins are 21-kDa membrane-associated guanine nucleotide-binding proteins [1] that alternate between an inactive form bound to guanosine diphosphate (GDP) and an active form bound to GTP (**Figure 2**) [2,3].

Mutations in *ras* is one of the most frequent aberrations in cancer and has been observed in ~ 30% of human cancers [2], with ~ 90% in pancreatic cancer [4], and in 10 – 65% of haematological malignancies [2,5]. Because farnesylation is the most important step in Ras activation [6], agents that block FTase have been developed to affect cancer cell survival and proliferation.

On activation, Ras stimulates the Raf/MEK/MAPK pathway, which induces an increase in the levels of cyclin D1 that promote the progression of cells through the G1 checkpoint and into S phase, leading to proliferation [7]. Activated Ras also stimulates PI3K/Akt and their downstream signalling pathways that stimulate cell

informa
healthcare

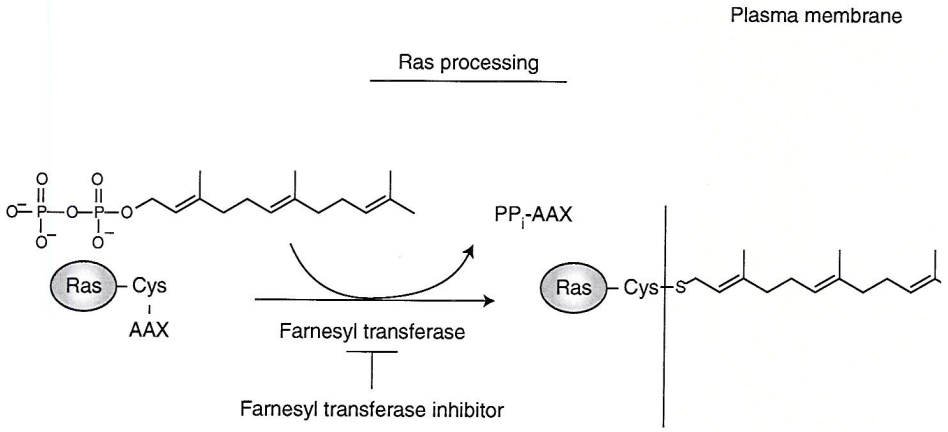


Figure 1. Mechanism of action of farnesyltransferase inhibitors. To become activated, Ras proteins must be localised to the inner side of the plasma membrane through a process called prenylation, which is the covalent addition of an isoprenoid moiety, farnesyl or geranylgeranyl issued from the cholesterol biosynthetic pathway to the C-terminal cysteine of substrate proteins. This process is catalysed by farnesyl- or geranylgeranyltransferase and occurs on the cysteine in a CAAX consensus sequence. In the CAAX sequence, the 'C' represents the cysteine, 'A' is an aliphatic amino acid and 'X' any amino acid. Once the prenyl group attaches to the CAAX moiety, the AAX part is cleaved. The C-terminal farnesyl-cysteine moiety is then carboxymethylated and a fatty acid palmitate residue is attached. This makes the Ras protein hydrophobic and facilitates its transfer to the cell membrane, where it becomes phosphorylated when activated by upstream tyrosine kinase signalling.
PP_i: Pyrophosphate group.

proliferation and survival [8]. In addition, Ras regulates Ral-GDS (Ras-related GTPase guanine nucleotide dissociation stimulator) proteins, guanine nucleotide exchange factors (GEFs) for Ral and phospholipase Cε (PLCε) that catalyse the hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol trisphosphate. Therefore, once in its active GTP-bound state, Ras can stimulate gene expression, cell-cycle progression and survival, cytoskeleton rearrangement, vesicle transport and Ca^{2+} signalling [7,8]. In addition, Ras interacts with specific integrin-α cytoplasmic domains and small GTP-binding protein, such as Rac and the Rho proteins, which may lead to an increase in the invasive capacity of neoplastic cells [7].

Substantial knowledge about the molecular biology of Ras and its downstream pathways has been obtained during the past decades. A series of recent studies has suggested that inhibition of the farnesylation of not-yet-identified protein/s other than Ras might be responsible for FTI effects. In this respect, RhoB, an important downstream effector of Ras, which affects various cellular processes – such as proliferation, apoptosis, actin formation, adhesion and motility – is also farnesylated as well as geranylgeranylated. This implies that FTI will also affect the function of this protein [9,10].

Moreover, Jiang *et al.* [11] found that the administration of a FTI to human cancer cells induces apoptosis through an inhibition of the PI3K/Akt2 cascade, but neither Ras nor RhoB (instead, an as-yet-unknown short-lived farnesylated protein) mediates this inhibition.

There is evidence that FTI might affect the activation of the centromere-binding proteins (CENP)-E and -F, which play an important role during mitosis by interfering with their prenylation [12].

Thus, although the Ras pathways have been investigated intensively, the complex biology and contribution of other main proteins to the cellular pharmacology of FTIs have probably not been sufficiently elucidated during the preclinical and clinical development of these anticancer drugs.

FTIs have demonstrated anticancer activity as single agents or in combination with standard radiation or cytotoxic chemotherapy [13-15]. In addition, FTIs have shown potential as a lung cancer chemopreventive agent [16]. Following the encouraging preclinical results, FTIs are now in clinical development.

On the basis of an understanding of the FTase reaction and the substrate specificity of the various enzymes, FTIs can be grouped into four main classes: farnesyl pyrophosphate analogues; CAAX peptidomimetics; bisubstrate inhibitors; and non-peptidomimetic inhibitors. FTIs compete with the CAAX-containing peptides for FTase binding sites. A total of three major nonpeptide FTIs have been developed and tested in clinical trials. Lonafarnib (SCH-66336) is an orally bioavailable tricyclic inhibitor of CAAX binding [17].

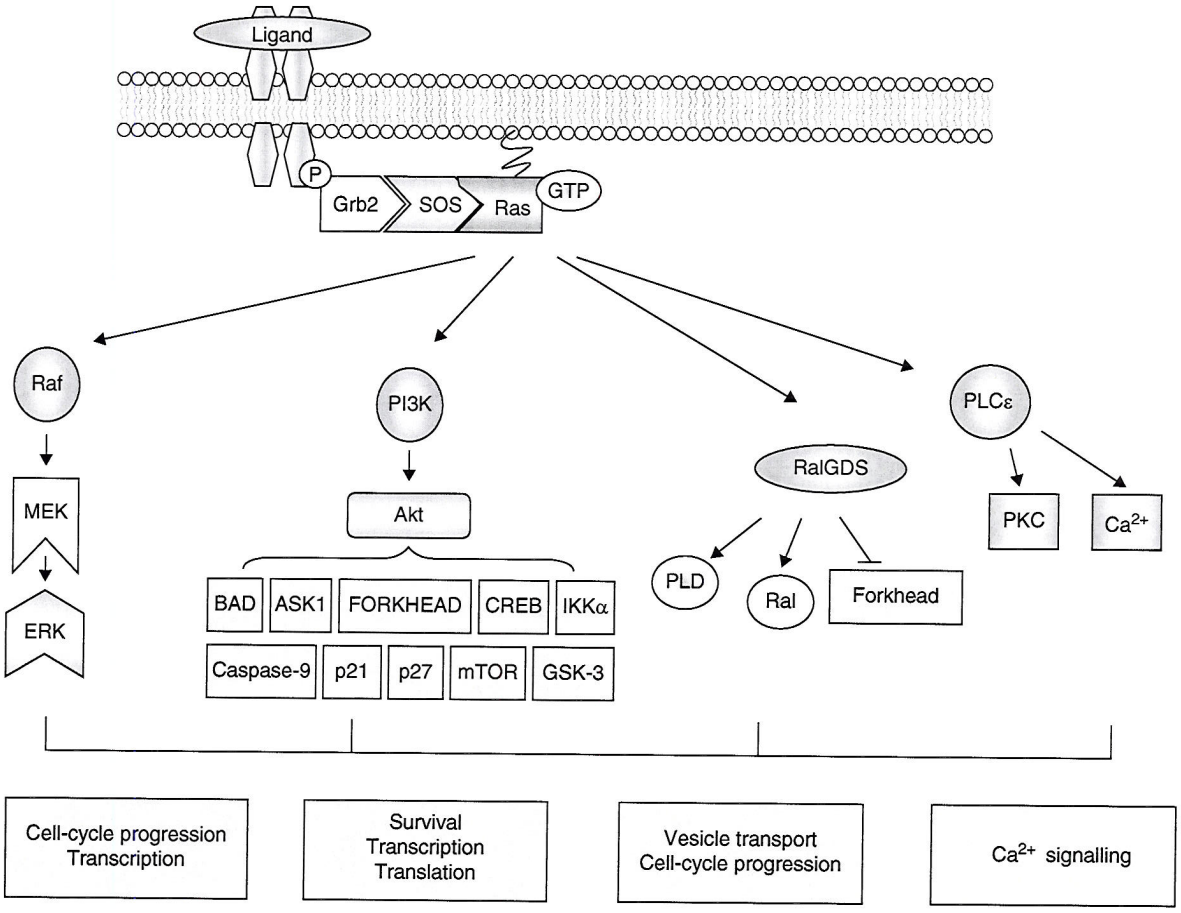


Figure 2. A simplified scheme of Ras-mediated signaling pathway. Once stimulated by extracellular signals (i.e. growth factor), tyrosine kinase receptors dimerise and autophosphotylate Src-homology-2 domains on the intracellular surface of the protein. This leads to binding of Grb-2, an adapter protein, and the SOS that interacts with Ras. The activated GTP-Ras is subsequently able to stimulate cell-proliferative and -survival processes through the activation of multiple pathways, such as Raf/MEK/Erk, PI3K/Akt/PKB, RalGDS (guanine nucleotide exchange factors for Ral) and PLCε, which activates PKC and mobilises Ca^{2+} from intracellular stores.
ERK: Extracellular-regulated kinase; Grb2: Growth factor receptor-bound protein 2; GSK3: Glycogen synthase kinase 3; MEK: Mitogen-activated kinase/ERK kinase; PKC: Protein kinase C; PL: Phospholipase; Ral: Ras-related GTPase; RalGD: Ral guanine nucleotide dissociation stimulator; SOS: Son of sevenless.

Lonafarnib has demonstrated activity against human tumour cell lines and xenografts. Tipifarnib (R-115777) is an orally active heterocyclic agent with an imidazole pharmacophore [18]. A third nonpeptide FTI in clinical trials is BMS-214662, an intravenously administered agent that induced apoptosis *in vitro* in several solid tumour cell types [19]. This review focuses on the pharmacological profile, preclinical and clinical activities, and toxicity spectrum of lonafarnib.

2. Preclinical activity

Lonafarnib has been known to inhibit anchorage-independent growth of K-*ras*-transformed rodent fibroblasts and of various mutated K-*ras*-containing human tumour cell lines with a median inhibitory concentration (IC_{50}) of 0.4 – 0.5 µmol/l [17,20]. Lonafarnib altered the cell-cycle distribution of several human tumour cell lines in two distinct ways. Most sensitive human tumour cell lines – such as HCT 116 (colon cancer cell line), NCI-H460 (lung cancer), McF7 (breast cancer) and MIA PaCa-2 (pancreatic cancer) – accumulated in the G2 – M phase after FTI treatment, but those with an activated H-*ras* (such as T 24 cell [bladder carcinoma]) accumulated in the G1 phase, suggesting that the biological effects induced by FTIs in cells with an activated H-*ras* are distinct from those in other sensitive cells [21]. Moreover, the cell-cycle blockade is often correlated with a p53-dependent p21 ($p21^{waf}$ or $p21^{Cip1}$) induction in many cell types [21].

Another important effect of lonafarnib is the induction of apoptosis notably at low serum concentrations [22]. Likewise, simultaneous treatment of H-*ras*-transformed fibroblasts with a MEK1 or -2 inhibitor markedly enhanced caspase-3

activity and the apoptotic response to lonafarnib [23]. Earlier reports [24,25] have shown the antileukaemic activity of lonafarnib in cell culture models of BCR-ABL transformation and in mouse models of BCR-ABL-positive leukaemia.

Lonafarnib has demonstrated excellent oral bioavailability and pharmacokinetic properties in *in vivo* studies, including mouse, rat and monkey systems [17]. Lonafarnib has shown growth-inhibitory effects on several human tumour xenografts, including DLD-1 and HCT16 colon carcinoma, A549 and HTB177 lung carcinoma, AsPc-1, HPAF-II, HS700T and MIA PaCa-2 pancreatic carcinoma, DU145 prostatic carcinoma and a H-*ras* transgenic mouse model [17]. In this study [17], the tumour regression that was observed after lonafarnib treatment in transgenic mice correlated with an increase in apoptosis in the tumour cells [17]. Enhanced efficacy was also observed when lonafarnib was combined with various cytotoxic agents (cyclophosphamide, 5-fluorouracil and vincristine). These collective findings indicate effective antitumour activity of lonafarnib.

Although FTIs were originally shown to inhibit farnesylation and activation of Ras, accumulating evidence suggests that lonafarnib suppresses anchorage-dependent and -independent growth of many human cancer cell lines, irrespective of whether they express wild-type or mutated Ras [17,26]. It has been suggested that RhoB (a protein that is prenylated) mediates the response to treatment with FTIs; RhoB can be farnesylated or geranylgeranylated and the antitumour effects of FTIs probably depend on the accumulation of geranylgeranylated forms of RhoB [9,10]. Lonafarnib also inhibits the PI3K/Akt-mediated growth and adhesion-dependent survival pathways and induces apoptosis via a Ras-independent mechanism [11]. In addition, lonafarnib has been demonstrated to block the prenylation of Rheb (a Ras homologue enriched in the brain), which is a small farnesylated GTPase that positively regulates mammalian target of rapamycin signalling and is generally higher in tumour cell lines than in normal cells [27]. Moreover, lonafarnib induces phosphorylation in head and neck squamous cell cancer (HNSCC) cell lines and, therefore, the inactivation of the eukaryotic translation elongation factor 2 (eEF2), which results in the suppression of protein synthesis [28]. Therefore, the lonafarnib-induced apoptotic activities that were shown in several human HNSCC cell lines could be due to the effects of the drug on protein synthesis. Indeed, several human cancer HNSCC cell lines treated with lonafarnib showed a reduction in the protein expression of antiapoptotic Bcl-2 and Bcl-XL and prosurivival Akt [29]. Recently, Takada *et al.* [30] have shown that lonafarnib inhibits activation of NF-κB and NF-κB-regulated gene expression induced by carcinogens and inflammatory stimuli identifying a new molecular basis for the suppression of proliferation and angiogenesis by SCH-66336.

Of interest, lonafarnib was shown to inhibit microtubule cytoskeleton, resulting in microtubule stabilisation and suppression of microtubule dynamics [31]. The microtubule-stabilising action of lonafarnib could be due to its effects on histone deacetylase 6 (HDAC6), the known tubulin deacetylase [31], or on CENPs [12]. These studies provided preclinical support for the following clinical studies [14,32] of the lonafarnib–paclitaxel combination that evidenced clinical activity with a good tolerability.

A recent study [33] has shown that lonafarnib has potential as an antiangiogenic therapeutic agent for aerodigestive tract tumours, a new role for this class of drugs. Lonafarnib appears to inhibit angiogenic activities of non-small cell lung cancer (NSCLC) and HNSCC cells by decreasing hypoxia- or IGF-stimulated HIF-1α expression by inhibiting the interaction between HIF-1α and heat-shock protein (Hsp)-90, thus causing the proteasomal degradation of HIF-1α and a decrease in vascular endothelial growth factor (VEGF) production from cancer and vascular endothelial cells. The action of lonafarnib seems to be mediated via an inhibition of tumour angiogenesis [33], leading to regression of orthotopic HNSCC on the tongue in mice. Tumour angiogenesis and metastases have been proposed to be associated with oncogenic *ras* mutations that mediate the increased expression of angiogenic growth factor and key metalloprotease, such as VEGF, gelatinase and stromelysin [34-36]. However, the antiangiogenic activities of lonafarnib in this study have also been documented as being Ras independent.

Despite diverse therapeutic actions of lonafarnib *in vitro*, ranging from antiangiogenic to antiproliferative and apoptotic effects on different types of cancer cells, it has shown therapeutic effects on a limited number of tumour types. Except for haematological malignancies, it appears to have only modest and variable antineoplastic effects on several types of tumours, probably because it stimulates alternative survival and antiapoptotic pathways. Therefore, it has been hypothesised that combination of lonafarnib with other targeted therapies that can block the alternative survival pathway could be a better therapeutic strategy to treat various malignancies. Indeed, the combination of lonafarnib and SCH58500 (a replication-deficient recombinant adenovirus that expresses the human p53 tumour suppressor) has shown synergistic or additive antiproliferative effects on a panel of tumour cell lines *in vitro* [37], and greater combined efficacy was observed *in vivo* in the DU145 human prostatic cancer and the *wap-ras/F* transgenic mouse cancer models [37]. Moreover, when the three-drug combination of lonafarnib, SCH58500 and paclitaxel was tested, each two-drug interaction displayed such marked synergy that the addition of a third drug to the statistical model only produced additivity [37]. Investigators have also looked at combinations of lonafarnib and IGFBP-3, a major serum IGF-binding protein that regulates IGF-mediated cancer cell survival and growth. Ras-mediated signalling has been suggested as interfering with antiproliferative effects of IGFBP-3 [38] and the enhancement of antitumour activity by the combination could have resulted from the blockade of Ras-mediated signalling by lonafarnib; however, the *ras* mutation does not seem to account for the increased apoptotic response of NSCLC cells

to combined treatment of IGFBP-3 and lonafarnib. Lonafarnib has also shown enhanced antitumour activities in several cancer cells *in vitro* and *in vivo* when combined with PI3K inhibitors, including LY-294002 and wortmannin [39]. Importantly, these PI3K inhibitors unmask the proapoptotic effects of FTIs in malignantly transformed cells, but not in normal cells. Lonafarnib has also shown enhanced antitumour activity when combined with cisplatin, taxanes and gemcitabine [7,40,41]; for example, lonafarnib sensitised tumour cells to paclitaxel-induced mitotic arrest [31], probably through its effects on HDAC6 [31] and CENPs [12]. Combined treatment with lonafarnib and the proteasome inhibitor bortezomib has also been tested in multiple myeloma cell lines whose growth depends on the Ras/Raf/MAPK pathway signalling [42]. This combination induced synergistic tumour cell death and an increase in the cleavage of caspase-3, -8 and -9 with concomitant downregulation of pAkt [42]. Recently, Nakajima *et al.* [43] showed that lonafarnib enhances the antiproliferative effects of imanitib against Bcr-Abl-expressing cells, which was consistent with the report of Hoover *et al.* [44]. Both groups have shown that treatment of the imanitib-resistant Bcr-Abl-expressing cells with imanitib plus lonafarnib leads to enhanced antiproliferative or proapoptotic effects.

3. Chemistry, pharmacokinetics and metabolism

Lonafarnib ([+]4-(2-[4-(8-chloro-3,*O*-dibromo-6,11-dihydro-5-benzocyclohepa[1,2-f]pyridin-11-yl)-1-piperidinyl]-2-oxoethyl)-1-piperidinecarboxamide is a tricyclic nonpeptidyl, nonsulfhydryl FTI (**Figure 3**). *In vitro*, it blocks farnesylation of H-*ras* with a IC_{50} of 1.9 nmol/l and inhibits the farnesylation of K-*ras*4B with a IC_{50} of 5.2 nmol/l. It does not inhibit geranylgeranyl protein transferase-1 in concentrations of ≤ 50 µmol/l [45]. The drug is absorbed relatively slowly and peak concentrations are generally reached at 2.7 – 8 h after drug administration. Peak plasma concentrations and area under the curve (AUC) values increase in a dose-proportional manner. An increase in dose from 25 to 400 mg was associated with an increase in mean peak plasma concentration by a factor of ~ 56 and in the AUC by a factor of ~ 200. The apparent clearance of lonafarnib decreases exponentially from 1190 ± 462 ml/min at a dose of 25 mg to 101 ± 27.3 ml/min at 400 mg and the volume of distribution ($V_{d,ss}$/F) decreases from 331 ± 27 l to 90.4 ± 22.4 l with the same doses. A trend of increasing plasma half-life with increasing dose that was statistically significant for lonafarnib 300 and 400 mg b.i.d. ($p < 0.007$; Kruskal–Wallis test) has been described [46]. The peak plasma concentrations and AUC_{0-12} increase by a factor of ~ 2 – 5 on repeated dosing in a dose-independent manner ($p = 0.103$; Kruskal–Wallis test), which is more than expected when only based on accumulation effects ($p = 0.0016$; paired student's t test). In contrast, the terminal disposition half-life is comparable between days 1 and 15 although the mean difference reached borderline significance ($p = 0.04$; Wilcoxon test for matched pairs of 10 patients). This suggests that the dose dependence in the apparent clearance does not arise primarily from factors associated with saturation of excretory routes. Steady-state concentrations of lonafarnib are attained by days 7 – 14 and there is only minor intrapatient variability in these levels [46]. The cumulative urinary excretion of unchanged lonafarnib was dose independent and accounted for < 0.02% of the administered dose. The mean renal clearance (the product of the dose fraction excreted unchanged in urine and the apparent total body clearance) is estimated as 0.117 ± 0.0105 ml/min, thus suggesting that lonafarnib is not cleared by renal processes [46].

4. Clinical efficacy and toxicity spectrum of lonafarnib

4.1 Phase I clinical trial

Several Phase I trials (**Table 1**) were performed with lonafarnib in patients who had advanced cancer. Adjei *et al.* [47] evaluated its effects in 20 patients by administering it orally twice daily for 7 days in a 21-day cycle. The most common haematological toxic effect was leucopoenia, which was usually associated with neutropoenia and was generally brief, mild and noncumulative. Diarrhoea was the principal non-haematological toxic effect; it was severe and dose limiting with lonafarnib 400 mg b.i.d. In a preliminary evaluation of drug activity, 1 patient who had metastatic NSCLC that had been refractory to other treatments presented a partial response after 2 courses of treatment and remained in the study for 14 months. Disease in another 8 patients was stable over 5 – 10 treatment cycles.

Lonafarnib has shown some toxicity in patients. The first clinical trial with lonafarnib in haematological malignancies was a Phase I study in 18 patients who had high-risk malignancies, including chronic myeloid leukaemia in the blastic phase (CML-BP), chronic myelomonocytic leukaemia (CMML), myelodysplastic syndrome (MDS), acute myelogenous leukaemia (AML) and acute lymphoblastic leukaemia (ALL) [48]. A starting dose of lonafarnib 100 mg b.i.d. p.o. was administered on a continuous schedule and the dose was increased until the maximum-tolerated dose (MTD) of 200 mg b.i.d. was reached. A total of 6 of 16 evaluable patients had a clinical response. Gastrointestinal toxicity, particularly diarrhoea (grade I/II), occurred in two-thirds of the patients. Farnesylation of the protein DNAJ was consistently inhibited.

A Phase Ib monotherapy study was undertaken in patients who had HNSCC. They were randomised to receive lonafarnib 100 – 300 mg b.i.d. or best supportive care for ≤ 14 days before surgery. Gastrointestinal side effects were observed but no dose-limiting toxicity (DLT) was reported. Partial responses were observed in 3 of 17 patients treated. Analysis of the surrogate marker DNAJ (Hdj-2), a

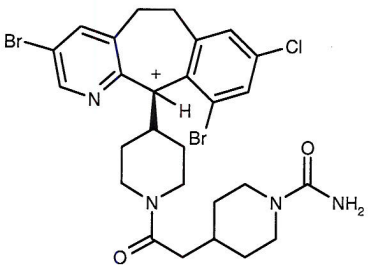


Figure 3. Chemical structure of lonafarnib.

farnesylated chaperone protein, in surgical samples revealed an increase in unfarnesylated protein in patients treated with lonafarnib [49].

A Phase I study [32] was conducted to establish the MTD of lonafarnib in combination with paclitaxel in patients who had solid tumours and to characterise the safety, tolerance, DLT and pharmacokinetics of the combination. Uninterrupted scheduled doses of lonafarnib 100, 125 and 150 mg b.i.d. p.o. were administered in combination with paclitaxel 135 or 175 mg/m^2 i.v. over 3 h on day 8 of every 21-day cycle. DLT consisted of grade 3 hyperbilirubinaemia, grade 4 diarrhoea, grade 3 peripheral neuropathy and grade 4 neutropoenia with fever. The MTDs established with the continual-reassessment method were lonafarnib 100 mg b.i.d. and paclitaxel 175 mg/m^2. A total of 21 patients were evaluable. Of 15 previously treated patients, 6 had durable partial responses, which included 2 of the 5 patients who had taxane-resistant metastatic NSCLC. A Phase I combination study [50] of continuous oral administration of lonafarnib with gemcitabine administered on days 1, 8 and 15 every 28 days has been reported in abstract form. The recommended doses for Phase II testing were either lonafarnib 150 mg in the morning and 100 mg in the evening with gemcitabine 1000 mg/m^2, or lonafarnib 200 mg b.i.d. with gemcitabine 600 mg/m^2. A total of two partial responses were documented in pancreatic cancer and one minor response each was documented in pancreatic cancer and pleural mesothelioma.

Hurwitz *et al.* [51] have also shown that 14 consecutive days of treatment with lonafarnib 200 mg b.i.d. p.o. (a recommended Phase II dose) induced nausea, diarrhoea and malaise as DLTs. Eskens *et al.* [46] used continuous oral administration of lonafarnib for 28 days and reported DLTs of grade 4 vomiting, grade 4 neutropoenia and thrombocytopenia, and grade 3 anorexia and diarrhoea; reversible grade 3 plasma creatinine increase occurred with lonafarnib 400 mg b.i.d. p.o. When the dose was reduced to 300 mg b.i.d., DLTs consisted of grade 4 neutropoenia, grade 3 neurocortical toxicity and grade 3 fatigue with grade 2 nausea and diarrhoea. No partial or complete responses were observed; 1 patient with pseudomyxoma peritonei had stable disease for > 9 months and 1 patient with metastatic follicular thyroid carcinoma had stable disease for 7 months with ongoing treatment. There were no partial or complete responses with the administration of lonafarnib 300 mg q.d. to 12 evaluable patients [52]. Haematological toxicity was not observed with lonafarnib 300 mg but became pronounced with 400 mg.

Lonafarnib has been investigated in eight and four patients with CML in chronic or accelerated phase, respectively, in whom imatinib therapy had been unsuccessful [53]. The patients received lonafarnib 200 mg b.i.d. continuously. A total of two patients had a haematological response. Diarrhoea was the most common adverse event, with grade 3 or 4 diarrhoea occurring in 4 patients.

4.2 Phase II/III clinical trial

Sharma *et al.* [54] conducted a Phase II trial in patients who had metastatic colorectal cancer that was considered to be refractory to first- and second-line therapy with 5-fluorouracil and irinotecan. The starting dose was lonafarnib 200 mg b.i.d. for 28 consecutive days. The major side effects were fatigue (grade 1 in 42% of the patients, grade 2 in 42% and grade 3 in 14%), diarrhoea (grade 1 in 23% and grade 3 in 42%) and nausea (grade 2 in 16%). In 19% of patients, grade 2 or 3 increases in serum creatinine concentration were observed and appeared to be related to dehydration induced by diarrhoea. Significant haematological toxicity was not observed. Pharmacological studies revealed adequate mean predose plasma concentrations in this group of patients on day 15 of therapy. In 21 evaluable patients, no objective responses were observed, although stable disease was seen in 3 patients for several months. Administration of lonafarnib was accompanied by gastrointestinal toxicity.

A recent Phase II study was conducted [55] to evaluate the antitumour activity of lonafarnib in patients who had urothelial cancer that was refractory to first-line cytotoxic treatment. Lonafarnib 200 mg b.i.d. p.o. was administered continuously in a 28-day cycle. In 19 eligible patients, drug-related grade 3 toxic effects included fatigue, anorexia, nausea, confusion, dehydration, muscle weakness, depression, headache and dyspnoea. A total of five patients discontinued the study protocol because of toxicity. No responses were observed in 10 evaluable patients. Of nine patients who were not evaluable, five had symptomatic progression and thus the study met multinomial criteria for stopping the study after the first stage.

An open-label Phase II, single-centre study [56] was conducted to determine the rate of objective responses to lonafarnib 200 mg b.i.d. to 15 patients with HNSCC that was refractory to chemotherapy, all of whom had received ≥ 1 platinum regimen but not > 3 courses of therapy. No objective response was observed; 7 patients maintained stable disease through ≥ 3 courses of therapy and 1 maintained stable disease for 8 courses (220 days). No treatment-related deaths were reported. Grade 4 hyperuricaemia occurred in 1 patient. Grade 3 adverse effects included anorexia, infection without

Table 1. Phase I and II trials of lonafarnib.

Study type	Tumour type	Schedule	Toxicity	Response	Ref.
Phase I					
Monotherapy	Solid tumour	25 – 400 mg b.i.d. on day 7 of every 21-day cycle	Leucopoenia with neutropoenia, diarrhoea, nausea and fatigue	1/20 PR in NSCLC; 8/20 SD	[47]
Monotherapy	CML-BP, CMML, MDS, AML and ALL	100 – 200 mg b.i.d. continuously	Nausea, diarrhoea and anorexia	6/16 clinical response (response rate: 38%)	[48]
Combination with paclitaxel	Solid tumours	100 – 150 mg b.i.d. continuously with paclitaxel 135 – 175 mg/m^2	Peripheral neuropathy, hyperbilirubinaemia, neutropoenia with fever, and diarrhoea	8/21 PR in NSCLC, salivary gland tumour; 7/21 SD	[32]
Combination with gemcitabine	Solid tumours	100 – 200 mg b.i.d. continuously with gemcitabine 600 – 1000 mg on days 1, 8 and 15 of every 28-day cycle	Nausea, vomiting, diarrhoea and myelosuppression	2/25 PR pancreatic cancer; 11/25 SD	[50]
Phase II					
Monotherapy	HNSCC	200 mg b.i.d.	Hyperuricaemia, anorexia, neutropoenia, QT elongation and diarrhoea	0/15 PR or CR, 7/15 SD	[56]
Monotherapy	MDS, CMML	200 – 300 mg b.i.d.	Diarrhoea and nausea	12/42 response (2CR and 10HI)	[57]
Combination with paclitaxel	NSCLC	100 mg b.i.d. with paclitaxel 175 mg/m^2 on days 1 and 8 of every 28-day cycle	Fatigue, diarrhoea, neutropoenia, dyspnoea and respiratory insufficiency	3/20 PR; 11/29 SD	[14]
Combination with gemcitabine	TCC	150 and 100 mg with gemcitabine 1000 mg on days 1, 8 and 15 of every 28-day cycle	Vomiting and diarrhoea	9/31PR and 1/31 CR	[59]

ALL: Acute lymphoblastic leukaemia; AML: Acute myelogenous leukaemia; CML-BP: Chronic myeloid leukaemia blast phase; CMML: Chronic myelomonocytic leukaemia; CR: Complete response; HI: Hazard index; HNSCC: head and neck squamous cell carcinoma; MDS: Myelodisplastic syndrome; NSCLC: Non-small cell lung cancer; PR: Partial response; SD: Standard deviation; TCC: Transitional cell cancer.

neutropoenia, cardiac toxicity (QTc interval elongation) and syncope in 1 patient each, respectively. No grade 3 or 4 haematological toxicity was reported, but grade 2 anaemia was noted in 2 patients. The most common toxic effects were grade 1 or 2 and were diarrhoea, nausea and fatigue in 10, 9 and 7 patients.

Results of a Phase II study in patients who had MDS (n = 32) or CMML (n = 35) were recently reported [57]. Patients received lonafarnib 200 – 300 mg b.i.d. p.o. Of 42 evaluable patients, 12 responded (4 MDS patients and 8 CMML patients), 2 of whom had complete response and 10 had a haematological improvement. In addition, 16 of 37 patients who had bone marrow (BM) blasts of 45% at baseline showed a reduction of ≥ 50% in BM blasts. Gastrointestinal toxicity, including diarrhoea and nausea, of grade 3 or 4 was observed in 17 patients (26%) who discontinued therapy.

The Phase I study [32] of combined therapy with lonafarnib and paclitaxel revealed manageable toxicity with evidence of clinical activity in heavily pretreated patients, some of whom had been resistant to each cytotoxic agent given alone. On the basis of the results of the Phase I study, a Phase II study of lonafarnib in combination with paclitaxel was conducted in patients who had metastatic (stage IIIB/V), or taxane-refractory or -resistant NSCLC to evaluate safety, tolerance and efficacy [14]. Patients received continuous lonafarnib 100 mg b.i.d. p.o. beginning on day 1 and paclitaxel 175 mg/m^2 i.v. over 3 h on day 8 of each 21-day cycle, and the MTD of this drug combination was as determined in the Phase I study. Of the 33 patients enrolled, 29 were evaluable for response. Partial response and stable disease were observed in 3 and 11 patients, respectively. Thus 14 patients experienced some clinical benefit. The updated and final median overall survival time was 39 weeks and the median progression-free survival time was 16 weeks. The combination of lonafarnib and paclitaxel was well tolerated with minimal toxicity and the results were not very different from those of single treatment; grade 3 toxicity included fatigue (9%), diarrhoea (6%) and dyspnoea (6%). Grade 3 neutropoenia occurred in only 1 patient. Grade 4 adverse events included respiratory insufficiency and acute respiratory failure in 2 and 1 patients, respectively.

The addition of lonafarnib to the combination of paclitaxel and carboplatin in patients with previously untreated advanced NSCLC did not produce a survival benefit in a recent Phase III study. In an interim analysis of 616 patients (of a total of 675 patients involved in this study), the overall survival was 144 days for patients treated with lonafarnib and 168 for patients who received placebo [58].

A Phase II study [59] of lonafarnib and gemcitabine combination therapy was conducted in patients who had been treated for advanced urothelial carcinoma. Patients were given a combination of lonafarnib 150 mg in the morning and 100 mg in the evening and gemcitabine 1000 mg/m^2 on days 1, 8 and 15 of each 28-day cycle. The 9 partial responses and 1 complete response in 31 assessable patients corresponded to an overall response rate of 32.3% (95% confidence interval [CI]: 17 – 51%). Lonafarnib had no effect on the pharmacokinetics of gemcitabine. In the combined treatment with gemcitabine, no patients had severe haematological toxicity, which was defined as grade 4 thrombocytopoenia or febrile neutropoenia. In another Phase II study [60], patients who had metastatic pancreatic cancer were randomised to lonafarnib 200 mg b.i.d. or gemcitabine 1000 mg/m^2/week for 7 weeks in an 8-week cycle. Drug activity was observed in both groups. A larger fraction of cases of stable or responsive disease was seen in the gemcitabine-treated group (40 versus 24%).

4.3 Safety and tolerance

The most frequent forms of non-haematological toxicity that have been reported for lonafarnib 200 mg b.i.d. were grade 1 or 2 fatigue (in ~ 47% of the patients), grade 1 or 2 diarrhoea (60 – 70%) and grade 1 – 2 nausea (2 – 16%). All of these were generally brief and reversible on discontinuation of the drug. Increased serum creatinine (grade 2 or 3) has been observed and appears to correlate with drug dosage; it is probably due to dehydration induced by diarrhoea. At higher dosages of lonafarnib, such as 400 mg b.i.d., grade 3 neurocortical toxicity has been described. Significant haematological toxicity with lonafarnib 200 mg b.i.d. has not been observed (only grade 1 thrombocytopoenia in ~ 20% of the patients and grade 2 or 3 anaemia in 20 – 30%).

Hyperleukocytosis complicating lonafarnib treatment has been reported in a recent clinical trial of lonafarnib in patients who had CMML [61]. Of 35 patients enrolled in the study, 15 experienced a rise in total leukocyte count to > 5000/µl/week. Of those patients, three developed rapid and progressive increases in white blood cells with lonafarnib treatment, associated with respiratory distress in two cases that resolved promptly after treatment with dexamethasone or withdrawal of lonafarnib. In two patients who presented with pulmonary infiltrates, infectious aetiology was excluded; cytological examination of the bronchoalveolar lavage fluid confirmed alveolar infiltration by mature monocytes. Patients with proliferative CMML in particular appear to be at significant risk for this complication.

5. Other FTIs

Among several inhibitors of the target enzyme (FTase) that have been developed, tipifarnib and lonafarnib have both entered Phase III clinical studies and are considered to be the two most promising oral FTIs. Tipifarnib was the first FTI tested in a clinical trial. In a Phase II trial in patients with advanced breast cancer, tipifarnib showed a significant activity with 9 partial responses and nine cases of stable disease (for ≥ 24 weeks) in 76 patients [13]. To date, the most promising activity of tipifarnib was reported in patients with untreated poor-risk acute myeloid leukaemia or MDS, in which a 33% response rate (8 complete and 2 partial responses) was seen [62]. Treatment with tipifarnib has been compared with placebo or standard therapies in at least two Phase III studies [18,63] and no significant antitumour effect was evidenced in both studies.

6. Expert opinion

Although contrasting results of clinical trials have moderated the initial enthusiasm for FTIs, they (and particularly lonafarnib) remain a promising class of novel antineoplastic agents. Lonafarnib monotherapy has important significant activity in haematological malignancies, even overcoming resistance to imanitib. In solid tumours, its activity seems to be modest and it should probably be studied in combination with cytotoxic agents, ionising radiation and other novel targeted drugs, such as antiangiogenic agents and IGFBP-3. FTIs were designed as Ras-blocking agents, but increasing lines of evidence indicate that other farnesylated targets are involved in their cytotoxic effects. The critical target proteins of FTIs and their mechanisms of action, particularly of their downstream effectors, are subjects of active investigation. Understanding those mechanisms will provide relevant biological markers that can help in the selection of patients who can benefit from FTI treatment.

Many clinical studies of combinations of FTIs and standard cytotoxic agents are underway. The toxic effects of the combinations appear to be manageable, and evidence of clinical activity in heavily pretreated patients, some of whom had been resistant to the cytotoxic agents when they were administered alone, has led to the consideration of FTIs as modulators of genotoxic agents, such as those that are used in standard chemotherapy and radiotherapy.

Acknowledgments

This work was supported by NIH grants RO1 CA100816 and R01 CA109520, American Cancer Society grant RSG-04-082-01-TBE and Department of Defense grant DAMD17-01-1-0689.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. BARBACID M: *Ras* genes. *Ann. Rev. Biochem.* (1987) **56**:779-827.
   - • **A comprehensive review.**
2. BEAUPRE DM, KURZROCK R: Ras and leukemia: from basic mechanisms to gene-directed therapy. *J. Clin. Oncol.* (1999) **17**(3):1071-1079.
3. McCORMICK F: Activators and effectors of ras p21 proteins. *Curr. Opin. Genet. Dev.* (1994) 4(1):71-76.
4. ALMOGUERA C, SHIBATA D, FORRESTER K *et al.*: Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell* (1988) **53**(4):549-554.
5. BOS JL: Ras oncogenes in human cancer: a review. *Cancer Res.* (1989) **49**(17):4682-4689.
6. KHOSRAVI-FAR R, COX AD, KATO K, DER CJ: Protein prenylation: key to ras function and cancer intervention? *Cell Growth Differ.* (1992) **3**(7):461-469.
7. ADJEI AA, DAVIS JN, BRUZEK LM, ERLICHMAN C, KAUFMANN SH: Synergy of the protein farnesyltransferase inhibitor SCH66336 and cisplatin in human cancer cell lines. *Clin. Cancer Res.* (2001) 7(5):1438-1445.
8. GILLE H, DOWNWARD J: Multiple ras effector pathways contribute to G_1 cell cycle progression. *J. Biol. Chem.* (1999) **274**(31):22033-22040.
   - •• **Identification of Ras effectors in regulating the cell-cycle machinery.**
9. LIU A, PRENDERGAST GC: Geranylgeranylated RhoB is sufficient to mediate tissue-specific suppression of Akt kinase activity by farnesyltransferase inhibitors. *FEBS Lett.* (2000) **481**(3):205-208.
10. LIU A, DU W, LIU JP, JESSELL TM, PRENDERGAST GC: RhoB alteration is necessary for apoptotic and antineoplastic responses to farnesyltransferase inhibitors. *Mol. Cell. Biol.* (2000) **20**(16):6105-6113.
11. JIANG K, COPPOLA D, CRESPO NC *et al.*: The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol. Cell. Biol.* (2000) **20**(1):139-148.
12. ASHAR HR, JAMES L, GRAY K *et al.*: Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. *J. Biol. Chem.* (2000) **275**(39):30451-30457.
13. JOHNSTON SR, HICKISH T, ELLIS P *et al.*: Phase II study of the efficacy and tolerability of two dosing regimens of the farnesyl transferase inhibitor, R115777, in advanced breast cancer. *J. Clin. Oncol.* (2003) **21**(13):2492-2499.
14. KIM ES, KIES MS, FOSSELLA FV *et al.*: Phase II study of the farnesyltransferase inhibitor lonafarnib with paclitaxel in patients with taxane-refractory/resistant nonsmall cell lung carcinoma. *Cancer* (2005) **104**(3):561-569.
   - •• **The first Phase II study showing objective response in NSCLC patients by combined treatment of lonafarnib and palitaxel.**
15. BERNHARD EJ, McKENNA WG, HAMILTON AD *et al.*: Inhibiting Ras prenylation increases the radiosensitivity of human tumor cell lines with activating mutations of *ras* oncogenes. *Cancer Res.* (1998) **58**(8):1754-1761.
16. ZHANG Z, WANG Y, LANTRY LE *et al.*: Farnesyltransferase inhibitors are potent lung cancer chemopreventive agents in A/J mice with a dominant-negative p53 and/or heterozygous deletion of Ink4a/Arf. *Oncogene* (2003) **22**(40):6257-6265.
17. LIU M, BRYANT MS, CHEN J *et al.*: Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and *wap-ras* transgenic mice. *Cancer Res.* (1998) **58**(21):4947-4956.
18. VAN CUTSEM E, VAN DE VELDE H, KARASEK P *et al.*: Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J. Clin. Oncol.* (2004) **22**(8):1430-1438.
19. TABERNERO J, ROJO F, MARIMON I *et al.*: Phase I pharmacokinetic and pharmacodynamic study of weekly 1-hour and 24-hour infusion BMS-214662, a farnesyltransferase inhibitor, in patients with advanced solid tumors. *J. Clin. Oncol.* (2005) **23**(11):2521-2533.
20. IZBICKA E, LAWRENCE R, DAVIDSON K *et al.*: Activity of a farnesyl transferase inhibitor (SCH66336) against a broad range of tumors taken directly from patients. *Proc. Am. Assoc. Cancer Res.* (1999) **40**:524 (Abstract 3454).
21. ASHAR HR, JAMES L, GRAY K *et al.*: The farnesyl transferase inhibitor SCH 66336 induces a $G_2 -> M$ or G_1 pause in sensitive human tumor cell lines. *Exp. Cell Res.* (2001) **262**(1):17-27.
22. SUN SY, ZHOU Z, WANG R, FU H, KHURI FR: The farnesyltransferase inhibitor lonafarnib induces growth arrest or apoptosis of human lung cancer cells without downregulation of Akt. *Cancer Biol. Ther.* (2004) **3**(11):1092-1098 (Discussion 1099-1101).
23. BRASSARD DL, ENGLISH JM, MALKOWSKI M *et al.*: Inhibitors of farnesyl protein transferase and MEK1,2 induce apoptosis in fibroblasts transformed with farnesylated but not geranylgeranylated H-*Ras*. *Exp. Cell Res.* (2002) **273**(2):138-146.
24. REICHERT A, HEISTERKAMP N, DALEY GQ, GROFFEN J: Treatment of Bcr/Abl-positive acute lymphoblastic leukemia in P190 transgenic mice with the farnesyl transferase inhibitor SCH66336. *Blood* (2001) **97**(5):1399-1403.
   - • **One of the first *in vivo* studies demonstrating the antileukaemic activity of lonafarnib.**
25. PETERS DG, HOOVER RR, GERLACH MJ *et al.*: Activity of the farnesyl protein transferase inhibitor SCH66336 against BCR/ABL-induced murine leukemia and primary cells from patients with chronic myeloid leukemia. *Blood* (2001) **97**(5):1404-1412.
26. FELDKAMP MM, LAU N, RONCARI L, GUHA A: Isotype-specific Ras. GTP-levels predict the efficacy of farnesyl transferase inhibitors against human astrocytomas regardless of *Ras* mutational status. *Cancer Res.* (2001) **61**(11):4425-4431.
   - • **The absence of *Ras* mutations does not preclude the chemotherapeutic efficacy of FTIs.**
27. BASSO AD, MIRZA A, LIU G *et al.*: The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity. *J. Biol. Chem.* (2005) **280**(35):31101-31108.
28. REN H, TAI SK, KHURI F, CHU Z, MAO L: Farnesyltransferase inhibitor SCH66336 induces rapid phosphorylation of eukaryotic translation elongation factor 2 in head and neck squamous cell carcinoma cells. *Cancer Res.* (2005) **65**(13):5841-5847.

▶29. CHUN KH, LEE HY, HASSAN K *et al.*: Implication of protein kinase B/Akt and Bcl-2/Bcl-XL suppression by the farnesyl transferase inhibitor SCH66336 in apoptosis induction in squamous carcinoma cells. *Cancer Res.* (2003) **63**(16):4796-4800.

▶30. TAKADA Y, KHURI FR, AGGARWAL BB: Protein farnesyltransferase inhibitor (SCH 66336) abolishes NF-kappaB activation induced by various carcinogens and inflammatory stimuli leading to suppression of NF-κB-regulated gene expression and up-regulation of apoptosis. *J. Biol. Chem.* (2004) **279**(25):26287-26299.

▶31. MARCUS AI, ZHOU J, O'BRATE A *et al.*: The synergistic combination of the farnesyl transferase inhibitor lonafarnib and paclitaxel enhances tubulin acetylation and requires a functional tubulin deacetylase. *Cancer Res.* (2005) **65**(9):3883-3893.

▶32. KHURI FR, GLISSON BS, KIM ES *et al.*: Phase I study of the farnesyltransferase inhibitor lonafarnib with paclitaxel in solid tumors. *Clin. Cancer Res.* (2004) **10**(9):2968-2976.

- **Evidence of clinical activity of lonafarnib and paclitaxel in NSCLC patients with taxane-resistant disease.**

▶33. HAN JY, OH SH, MORGILLO F *et al.*: Hypoxia-inducible factor 1α and antiangiogenic activity of farnesyltransferase inhibitor SCH66336 in human aerodigestive tract cancer. *J. Natl. Cancer Inst.* (2005) **97**(17):1272-1286.

- ***In vitro* study showing the antiangiogenic effects of lonafarnib.**

▶34. SU ZZ, AUSTIN VN, ZIMMER SG, FISHER PB: Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-*ras*-transformed cloned rat embryo fibroblast cells. *Oncogene* (1993) **8**(5):1211-1219.

▶35. ZABRENETZKY V, HARRIS CC, STEEG PS, ROBERTS DD: Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. *Int. J. Cancer.* (1994) **59**(2):191-195.

▶36. RAK J, MITSUHASHI Y, BAYKO L *et al.*: Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res.* (1995) **55**(20):4575-4580.

▶37. NIELSEN LL, SHI B, HAJIAN G *et al.*: Combination therapy with the farnesyl protein transferase inhibitor SCH66336 and SCH58500 (p53 adenovirus) in preclinical cancer models. *Cancer Res.* (1999) **59**(23):5896-5901.

- **Study showing the synergistic combination of FTIs with p53 adenovirus.**

▶38. MARTIN JL, BAXTER RC: Oncogenic ras causes resistance to the growth inhibitor insulin-like growth factor binding protein-3 (IGFBP-3) in breast cancer cells. *J. Biol. Chem.* (1999) **274**(23):16407-16411.

- **Implication of Ras-mediated signaling as a resistance mechanism to IGFBP-3 treatment.**

▶39. EDAMATSU H, GAU CL, NEMOTO T, GUO L, TAMANOI F: Cdk inhibitors, roscovitine and olomoucine, synergize with farnesyltransferase inhibitor (FTI) to induce efficient apoptosis of human cancer cell lines. *Oncogene* (2000) **19**(27):3059-3068.

▶40. SUN J, BLASKOVICH MA, KNOWLES D *et al.*: Antitumor efficacy of a novel class of non-thiol-containing peptidomimetic inhibitors of farnesyltransferase and geranylgeranyltransferase I: combination therapy with the cytotoxic agents cisplatin, Taxol, and gemcitabine. *Cancer Res.* (1999) **59**(19):4919-4926.

- ***In vitro* study of the combination of lonfarnib and cytotoxic agents.**

▶41. LOPREVITE M, FAVONI RE, DE CUPIS A *et al.*: *In vitro* study of farnesyltransferase inhibitor SCH 66336, in combination with chemotherapy and radiation, in non-small cell lung cancer cell lines. *Oncol. Rep.* (2004) **11**(2):407-414.

▶42. DAVID E, SUN SY, WALLER EK *et al.*: The combination of the Farnesyl transferase inhibitor (lonafarnib) and the proteasome inhibitor (bortezomib) induces synergistic apoptosis in human myeloma cells that is associated with down-regulation of p-AKT. *Blood* (2005) **106**(13):4322-9.

- ***In vitro* study of the combined treatment of lonafarnib and the proteasome inhibitor bortezomib.**

▶43. NAKAJIMA A, TAUCHI T, SUMI M, BISHOP WR, OHYASHIKI K: Efficacy of SCH66336, a farnesyl transferase inhibitor, in conjunction with imatinib against BCR-ABL-positive cells. *Mol. Cancer Ther.* (2003) **2**(3):219-224.

- **Role of lonafarnib in enhancing the antiproliferative effects of imatinib.**

▶44. HOOVER RR, MAHON FX, MELO JV, DALEY GQ: Overcoming STI571 resistance with the farnesyl transferase inhibitor SCH66336. *Blood* (2002) **100**(3):1068-1071.

▶45. BISHOP WR, BOND R, PETRIN J *et al.*: Novel tricyclic inhibitors of farnesyl protein transferase. Biochemical characterization and inhibition of Ras modification in transfected Cos cells. *J. Biol. Chem.* (1995) **270**(51):30611-30618.

▶46. ESKENS FA, AWADA A, CUTLER DL *et al.*: Phase I and pharmacokinetic study of the oral farnesyl transferase inhibitor SCH 66336 given twice daily to patients with advanced solid tumors. *J. Clin. Oncol.* (2001) **19**(4):1167-1175.

▶47. ADJEI AA, ERLICHMAN C, DAVIS JN *et al.*: A Phase I trial of the farnesyl transferase inhibitor SCH66336: evidence for biological and clinical activity. *Cancer Res.* (2000) **60**(7):1871-1877.

48. LIST AF, DEANGELO D, O'BRIEN S *et al.*: Phase I study of continuous oral administration of lonafarnib (Sarasar) in patients with advanced hematologic malignancies. *Blood* (2002) **100**:789a.

- **The first Phase I clinical study in haematological malignancies.**

49. KIES MS, CLAYMAN GL, El-NAGGAR AK *et al.*: Induction therapy with SCH 66336, a farnesyltransferase inhibitor, in squamous cell carcinoma (SCC) of the head and neck. *Proc. Ann. Meet. Am. Soc. Clin. Oncol.* (2001) **20**:896 (Abstract).

- **Phase I study in HNSCC.**

50. HURWITZ H, AMADO R, PRAGER D *et al.*: Phase I and clinical pharmacokinetic trial of the farnesyl transferase inhibitor SCH66336 plus gemcitabine in advanced cancer. *Proc. Am. Soc. Clin. Oncol.* (2000):185a (Abstract 717).

- **Evidence of clinical activity of lonafarnib–gemcitabine in pancreatic cancer.**

51. HURWITZ H, COLVIN OM, PETROS WP *et al.*: Phase I and pharmacokinetic study of SCH66336, a novel FPTI, using a 2-week on, 2-week off schedule. *Proc. Am. Soc. Clin. Oncol.* (1999) **18**:156a.

▶52. AWADA A, ESKENS FA, PICCART M *et al.*: Phase I and pharmacological study of the oral farnesyltransferase inhibitor SCH 66336 given once daily to patients with advanced solid tumours. *Eur. J. Cancer* (2002) **38**(17):2272-2278.

▶53. CORTES J, DALEY GQ, TALPAZ M *et al.*: Pilot study of SCH66336 a farnesyl transferase inhibitor in patients with chronic or accelerator phase resistant or refractory to imanitib. *Blood* (2002) **100**:164a.

▶54. SHARMA S, KEMENY N, KELSEN DP *et al.*: A Phase II trial of farnesyl protein transferase inhibitor SCH 66336, given by twice-daily oral administration, in patients with metastatic colorectal cancer refractory to 5-fluorouracil and irinotecan. *Ann. Oncol.* (2002) **13**(7):1067-1071.

•• **Phase II study of single-agent lonafarnib in colorectal cancer patients; no objective responses were observed.**

▶55. WINQUIST E, MOORE MJ, CHI KN *et al.*: A multinomial Phase II study of lonafarnib (SCH 66336) in patients with refractory urothelial cancer. *Urol. Oncol.* (2005) **23**(3):143-149.

56. YANG H, KIES MS, GLISSON B *et al.*: A Phase II study of Lonafarnib (SCH66336) in patients with chemo-refractory advanced head and neck squamous cell carcinoma (HNSCC). *Proc. Am. Soc. Clin. Oncol.* (2000) (Abstract 5565 C).

• **Phase II study of single-agent lonafarnib in HNSCC patients; no objective responses were observed.**

▶57. FELDMAN E, CORTES J, HOLYOAK T *et al.*: Continuous oral lonafarnib (Sarasar) for the treatment of patients with myelodysplastic syndrome. *Blood* (2003) **102**:421a.

58. BLUMENSCHEIN G, LUDWIG C, THOMAS G *et al.*: A randomized Phase III trial comparing lonafarnib/carboplatin/paclitaxel versus carboplatin/paclitaxel (CP) in chemotherapy-naive patients with advanced or metastatic non-small cell lung cancer (NSCLC). *Proceedings from the 11th World Conference on Lung Cancer*, Barcelona, Spain (2005) (Abstract O-082).

▶59. THEODORE C, GEOFFROIS L, VERMORKEN JB *et al.*: Multicentre EORTC study 16997: feasibility and Phase II trial of farnesyl transferase inhibitor and gemcitabine combination in salvage treatment of advanced urothelial tract cancers. *Eur. J. Cancer* (2005) 41(8):1150-1157.

• **Phase II study evidencing objective responses by combined treatment of lonafarnib and gemcitabine in patients with advanced urothelial carcinoma.**

60. LERSCH C, VAN CUSTEM E, AMADO R *et al.*: Randomized Phase II study of SCH 66336 and gemcitabine in the treatment of metastatic adenocarcinoma of the pancreas. *Proc. Ann. Meet. Am. Soc. Clin. Oncol.* (2001) **20**:608.

• **Phase II study evidencing objective responses with the combined treatment of lonafarnib and gemcitabine in patients with metastatic pancreatic cancer.**

▶61. BURESH A, PERENTESIS J, RIMSZA L *et al.*: Hyperleukocytosis complicating lonafarnib treatment in patients with chronic myelomonocytic leukemia. *Leukemia* (2005) **19**(2):308-310.

▶62. LANCET JE, KARP JE, GOTLIB L *et al.*: Zarnestra™ (R115777) in previously untreated poor-risk AML and MDS: preliminary results of a Phase II trial. *Blood* (2002) **100**:560a.

▶63. RAO S, CUNNINGHAM D, DE GRAMONT A *et al.*: Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *J. Clin. Oncol.* (2004) **22**:3950-3957.

Affiliation

Floriana Morgillo MD & Ho-Young Lee† PhD
†Author for correspondence
M. D. Anderson Cancer Center, Department of Thoracic/Head & Neck Medical Oncology, Houston, TX, USA
Tel: +1 713 792 6363; Fax: +1 713 796 8655;
E-mail: hlee@mdanderson.org

Involvement of Mitochondrial and Akt Signaling Pathways in Augmented Apoptosis Induced by a Combination of Low Doses of Celecoxib and *N*-(4-Hydroxyphenyl) Retinamide in Premalignant Human Bronchial Epithelial Cells

Claudia P. Schroeder, Humam Kadara, Dafna Lotan, Jong K. Woo, Ho-Young Lee, Waun Ki Hong, and Reuben Lotan

Department of Thoracic, Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Celecoxib is being evaluated as a chemopreventive agent. However, its mechanism of action is not clear because high doses were used for *in vitro* studies to obtain antitumor effects. We found that celecoxib inhibited the growth of premalignant and malignant human bronchial epithelial cells with IC_{50} values between 8.9 and 32.7 μmol/L, irrespective of cyclooxygenase-2 (COX-2) expression. Normal human bronchial epithelial cells were less sensitive to celecoxib. Because these concentrations were higher than those attainable *in vivo* (≤5.6 μmol/L), we surmised that combining celecoxib with the synthetic retinoid *N*-(4-hydroxyphenyl) retinamide (4HPR) might improve its efficacy. Treatment of premalignant lung cell lines with combinations of clinically relevant concentrations of celecoxib (≤5 μmol/L) and 4HPR (≤0.25 μmol/L) resulted in greater growth inhibition, apoptosis induction, and suppression of colony formation than did either agent alone. This combination also decreased the levels of Bcl-2, induced the release of mitochondrial cytochrome *c*, activated caspase-9 and caspase-3, and induced cleavage of poly(ADP-ribose)polymerase at concentrations at which each agent alone showed no or minimal effects. Furthermore, combinations of celecoxib and 4HPR suppressed the phosphorylation levels of serine/threonine kinase Akt and its substrate glycogen synthase kinase-3β more effectively than the single agents did. Accordingly, overexpression of constitutively active Akt protected bronchial epithelial cells from undergoing apoptosis after incubation with both celecoxib and 4HPR. These findings indicate that activation of the mitochondrial apoptosis pathway and suppression of the Akt survival pathway mediate the augmented apoptosis and suggest that this combination may be useful for lung cancer chemoprevention. (Cancer Res 2006; 66(19): 9762-70)

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). The overall survival rate is poor and has not changed appreciably for several decades despite the introduction of novel agents and combined treatment modalities using surgery, radiotherapy, and chemotherapy. Therefore, new strategies are needed for intervention at early stages of lung carcinogenesis before malignant tumors become clinically evident. One of the promising approaches to accomplishing this goal is cancer chemoprevention (2, 3). In fact, high-risk populations for developing lung cancer, including former and current smokers, represent suitable candidates for chemoprevention trials (4). However, randomized controlled trials using dietary supplementation to prevent lung cancer in smokers have shown rather disappointing results (5) and have highlighted the necessity to find novel agents and combination strategies.

Nonsteroidal, anti-inflammatory drugs have been observed to reduce the relative risk for tobacco-induced lung carcinogenesis in both preclinical and clinical studies (4, 6). The anti-inflammatory action of these drugs is mediated through their inhibitory effect on cyclooxygenases (COX), which are essential enzymes for the synthesis of prostaglandins generated from arachidonic acid (7). In fact, COX-2 isoenzyme is frequently up-regulated in neoplastic tissue of the lung and seems to be associated with a poor prognosis among patients with non–small cell lung cancer, implicating a role in carcinogenesis (8, 9). Celecoxib, the first selective COX-2 inhibitor approved for chemoprevention of colon cancer in patients with familial adenomatous polyposis (10), has also been found to decrease the incidence of esophageal cancer in humans (11), and colon (12), gastric (13), lung (14, 15), mammary (16), oral (17), prostate (18), urinary bladder (19), and skin (20) cancer in various animal models with no associated toxicity. Moreover, a number of studies have shown that celecoxib at clinically feasible concentrations (≤5.6 μmol/L) markedly suppresses the biosynthesis of PGE_2 in COX-2–expressing lung cancer cells (21, 22). However, the fact that much higher doses of celecoxib (≥25 μmol/L) are required for growth inhibition and apoptosis induction in cell culture systems suggests a mode of action independent of COX-2–inhibitory activity and raises questions about the clinical relevance of *in vitro* findings (23). Additionally, data have shown that celecoxib was similarly effective in distinct types of cancer cells that were negative for COX-2 expression (24, 25). In short, the precise molecular mechanisms underlying the antitumor effects of celecoxib is not fully understood.

A strong rationale exists for the use of combinations of agents that act in an additive or synergistic manner by increasing treatment efficacy and/or decreasing drug toxicity (5). We previously showed that *N*-(4-hydroxyphenyl) retinamide (4HPR; fenretinide), a synthetic derivative of retinoic acid, exerts potent proapoptotic effects on a variety of cancer cells (26, 27). Moreover, 4HPR

Note: W.K. Hong is an American Cancer Society Clinical Research Professor. R. Lotan is the incumbent of the Irving and Nadine Mansfield and Robert David Levitt Cancer Research Chair.

Requests for reprints: Reuben Lotan, Department of Thoracic, Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center; 1515 Holcombe Boulevard, P.O. Box 432, Houston, TX 77030. Phone: 713-792-8467; Fax: 713-745-5656; E-mail: rlotan@mdanderson.org.

doi:10.1158/0008-5472.CAN-05-4124

combined with celecoxib inhibited growth and induced apoptosis of non–small cell lung cancer cell lines more efficiently than either agent alone did (25), suggesting further investigations for the treatment of human lung cancer. Supported by findings of preclinical and clinical cancer chemoprevention trials, which have indicated great promise for 4HPR and celecoxib administered as single agents (28, 29), we asked whether the combination of both would enhance their individual effects in an *in vitro* model of tobacco-induced human lung carcinogenesis. We found that celecoxib combined with 4HPR at clinically attainable concentrations inhibited growth and induced apoptosis of premalignant and tumorigenic bronchial epithelial cell lines by activating the mitochondrial apoptosis pathway as well as suppressing the Akt survival pathway.

Materials and Methods

Reagents. Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-] benzene-sulfonamide) was obtained from GD Searle & Co (Chicago, IL), and 4HPR was kindly provided by Dr. James Zweibel. The stock solutions of celecoxib (0.05 mol/L) and 4HPR (0.01 mol/L), both in DMSO, were stored at −80°C and diluted to the desired concentrations with culture medium before use. Bovine serum albumin, DMSO, EDTA, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). PBS and trypsin were from Life Technologies Invitrogen Corporation (Carlsbad, CA). All culture plasticwares were obtained from BD Bioscience Labware (Bedford, MA).

***In vitro* model of human lung carcinogenesis.** The cell lines used in this study represent an *in vitro* model of human lung carcinogenesis. BEAS-2B is a human bronchial epithelial cell line immortalized using an adenovirus 12-SV40 hybrid virus. The transformed 1198 and the tumorigenic 1170-I cell lines were derived from BEAS-2B by exposure to cigarette smoke condensate *in vivo* after transplantation into nude mice (30). The immortalized 1799 cell line was derived from BEAS-2B cells by *in vivo* transplantation without exposure to cigarette smoke condensate (31). These cell lines were obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (San Diego, CA) and used at the second passage only. The non–small cell lung cancer cell line A549 (American Type Cell Culture Collection, Rockville, MD) was included as a positive control for COX-2 expression (25). BEAS-2B and 1799 cells were grown in keratinocyte serum-free medium (K-SFM) containing human recombinant epidermal growth factor (2.5 μg) and bovine pituitary extract (25 mg; Life Technologies Invitrogen Corporation) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cell lines 1198 and 1170-I were maintained in K-SFM supplemented with 3% fetal bovine serum (FBS) from HyClone Laboratories, Inc. (Logan, UT).

Cell growth studies. The cell lines cultured in the medium described above were seeded into 96-well culture plates (6×10^3 or 2×10^4 per well for confluent NHBE culture) in K-SFM with and without 3% FBS, allowed to adhere overnight at 37°C, followed by treatment with celecoxib, 4HPR, and their combinations for 3 days. Control cultures were incubated with DMSO alone. An automated plate reader (model MR5000, Dynatech Laboratories Inc., Chantilly, VA) was used to estimate cell numbers using the sulforhodamine B assay (32). The inhibition of cell growth was calculated as $(1 - A_t / A_c) \times 100\%$, where A_t and A_c represent absorbencies of treated and control cultures, respectively. Concentration response curves were plotted, and IC_{50} concentrations of celecoxib were calculated by interpolation after 3 days in the presence or absence of 3% FBS.

Colony formation studies. Exponentially growing cells were seeded into six-well culture plates (0.8×10^5/well) overnight before treatment with celecoxib, 4HPR, and their combinations. The medium was removed and replaced with fresh medium containing these agents every 3 days. After 14 days of incubation, the cells were fixed with methanol/acetic acid (3:1, v/v) and stained with crystal violet in methanol (0.5%, v/v) to visualize the colonies. For quantification, each well was divided into eight fields, and the number of colonies ≥1 mm was estimated with a colony counter (Fisher Scientific, Pittsburgh, PA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Fragmentation of intranucleosomal DNA was evaluated using an apoptosis *in situ* detection kit (Apo-Direct, Phoenix Flow Systems, Inc., San Diego, CA) based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (33). After treatment, both adherent and nonadherent cells were harvested by trypsinization, pelleted by centrifugation, fixed with ice-cold ethanol (70%, v/v), and stained according to the protocol of the manufacturer. Fluorimetric measurement and data analysis were done on a Coulter XL flow cytometer (Miami, FL). The percentage of cells that were apoptotic was determined from the proportion of fluorescein-isothiocyanate–positive cells within 10,000 cells analyzed. Two independent experiments were done.

Western blot analysis. Samples containing 50 μg of total cellular protein mixed in sample buffer [0.5 mol/L Tris (pH 6.8), 0.3% glycerol, 0.03% β-mercaptoethanol, 10% SDS, and 0.001% bromphenol blue] were electrophoretically separated through 8% to 12% SDS-polyacrylamide slab gels, followed by transfer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Briefly, cell monolayers were washed twice with ice-cold PBS and collected in lysis buffer containing 150 mmol/L NaCl, 0.02% NaN_3, 2% Igepal CA-630, 0.5% sodium deoxycholate, 0.2% SDS, and 50 mmol/L Tris-HCl (pH 8.0) supplemented with the protease inhibitors leupeptin (1 μg/mL), aprotinin (1 μg/mL), pepstatin (0.5 μg/mL), and phenylmethylsulfonyl fluoride (100 μg/mL). Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories). After blocking with 3% nonfat dry milk solution in 0.1% (w/v) Tween 20 in PBS, the membranes were probed with antihuman antibodies at appropriate dilutions against COX-2 (Oxford Biomedical Research, Inc., Oxford, MI); caspase-3 (clone 31A1067, Imgenex, San Diego, CA); caspase-8, Bcl-2, Bcl-x_L, Bcl-x_S, and Bax (all from Santa Cruz Biotechnology, Santa Cruz, CA); poly(ADP-ribose) polymerase (PARP); caspase-9; hemagglutinin tag for the recombinant protein (HA-tag 262K); and antibodies included in the phosphorylated Akt pathway sampler kit (all from Cell Signaling Technology, Inc., Charlottesville, VA). Antibody binding was detected with horseradish peroxidase–linked secondary antibody and enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). Loading and transferring control was confirmed by probing the membranes with anti-β-actin antibody or staining with Ponceau S solution (Sigma Chemical). Mitochondria and cytosol fractionation was done according to a cytochrome *c* releasing apoptosis assay kit (BioVision, Inc., Mountain View, CA).

Adenoviral vector generation. An adenoviral vector expressing constitutively active Akt (MyrAkt), referred to here as Ad-MyrAkt, and an adenoviral vector expressing empty vector, referred to here as Ad-EV, were amplified as described previously (34). Briefly, 1799 transformed cells were infected at 5×10^3 multiplicity of infection (MOI) with Ad-MyrAkt and Ad-EV, respectively. After 2 hours, the medium was replaced with fresh medium containing celecoxib and 4HPR followed by 2 days of incubation. The adenoviral vector expressing a full-length human Akt1 with the Src myristoylation signal fused in-frame to the c-Akt coding sequence with HA under the control of the cytomegalovirus promoter (Ad5CMV-MyrAkt-HA) was constructed using the pAd-shuttle vector system (35, 36). The presence of MyrAkt-HA was confirmed by Western blot analysis of Akt and HA expression. The activity of Ad5CMV-MyrAkt-HA was assessed by detection of cleavage of PARP (Cell Signaling Technology), activation of caspase-3 (Imgenex), and expression of Bcl-2 (Cell Signaling Technology) using immunoblotting.

Statistical analysis. Growth studies and the number of colonies were analyzed for statistical significance using Student's two-tailed *t* test with $P \leq 0.05$ and $P \leq 0.005$, respectively. The results represent mean values ± SD of three independent experiments, each done in quadruplicate.

Results

Celecoxib inhibits growth of human premalignant and malignant lung cell lines independent of COX-2. To determine whether the growth-inhibitory effects of celecoxib in an *in vitro*

model of human lung carcinogenesis were associated with the expression of the presumed target enzyme, we analyzed the constitutive COX-2 levels of cells. COX-2 was expressed at a very low level in NHBE cells; was not detected in BEAS-2B, 1799, 1198, or 1170-I cells; and was not induced in 1198 or 1170-I cell lines whether they were cultured in the presence or absence of 3% FBS (Fig. 1*A*). However, the robust expression of COX-2 in A549 lung cancer cells, which were included as a positive control for COX-2, increased by ~20% when grown in the absence of FBS. Furthermore, the constitutive levels of PGE_2 in BEAS-2B, 1198, and A549 cells were 0.2, 0.04, and 2.7 ng/10^6 cells, respectively, as determined by electrospray ionization liquid chromatography tandem mass spectrometry (24), correlated with their differential COX-2 expression. As shown in Fig. 1*B*, celecoxib inhibited the growth of premalignant (BEAS-2B, 1799, and 1198) and tumorigenic 1170-I cell lines with IC_{50} values ranging from 8.9 to 10.1 μmol/L in K-SFM and from 15.9 to 18.7 μmol/L in 3% FBS-containing K-SFM. A549 lung cancer cells were ~2-fold less sensitive to celecoxib than premalignant cell lines irrespective of the presence or absence of serum. NHBE cells, which were seeded at subconfluent density to allow them to proliferate, exhibited a lower sensitivity to celecoxib characterized by IC_{50} values of 23.2 and 62 μmol/L in the absence and presence of 3% FBS, respectively. Confluent cultures of NHBE cells, which better mimic the nonproliferative state *in vivo*, were less sensitive to celecoxib with IC_{50} values of 29.2 and 80 μmol/L in serum-free and serum-containing medium, respectively. Thus, the growth-inhibitory activity of celecoxib seemed to be independent of COX-2 expression and activity. In addition, COX-2 was not modulated in 1198 cells cultured in standard medium or medium supplemented with celecoxib (2.5 and 5 μmol/L), 4HPR (0.25 μmol/L), or both, as shown by immunoblotting (Fig. 1*C*).

Celecoxib plus 4HPR is more potent than either agent alone in inhibiting growth of premalignant and tumorigenic lung cell lines. The effects of low doses of celecoxib in combination with the proapoptotic retinoid 4HPR on cell growth were studied because the IC_{50} values determined in the previous experiment were at least 3-fold higher than peak plasma levels obtained in patients treated with celecoxib (23) and because our recent studies on various non–small cell lung cancer cell lines showed that simultaneous treatment of celecoxib with 4HPR resulted in additive growth suppression (25). As shown in Fig. 2, combinations of 5 μmol/L celecoxib with 0.2 μmol/L 4HPR inhibited cell growth of premalignant and tumorigenic cell lines more effectively than treatment with either agent alone ($P \leq 0.05$ and $P \leq 0.005$ compared with untreated cultures). Furthermore, combinations of 2.5 μmol/L celecoxib and 0.2 μmol/L 4HPR showed greater growth suppression in 1799, 1198, and 1170-I than in BEAS cells. In contrast, NHBE cells grown *in vitro* under either subconfluent or confluent condition seemed to be sensitive to the growth-inhibitory effects of 4HPR (0.2 μmol/L), whereas celecoxib showed no significant activity at concentrations up to 5 μmol/L. Interestingly, combinations of the two agents were less or similarly effective than treatment of NHBE cells with each agent alone.

Celecoxib plus 4HPR suppresses colony formation of premalignant and tumorigenic lung cell lines more effectively than either agent alone. Because the growth-inhibitory effects induced by celecoxib, 4HPR, or both in monolayer cultures were modest, we asked whether treatment with either or both agents could exert greater activity on the formation of colonies, which allows an investigation over a longer period of time. As single

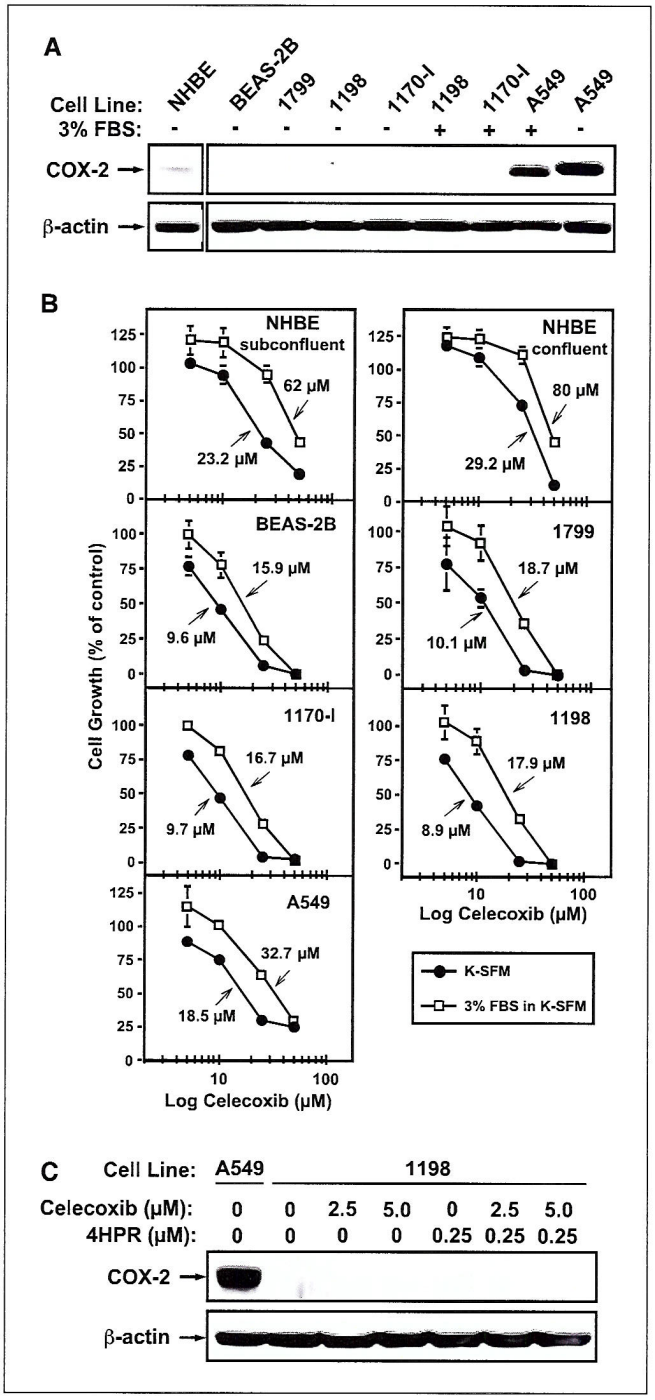


Figure 1. Expression of COX-2 in various premalignant, normal, and malignant human lung cell lines and their growth inhibition by celecoxib. *A,* human normal (NHBE), immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I and A549) epithelial cells were grown in K-SFM supplemented with epidermal growth factor (2.5 μg) and bovine pituitary extract (25 mg) in the presence or absence of FBS (3%). The cells were harvested after 5 days, lysed, and subjected to Western blot analysis (50 μg/lane) using anti-human COX-2 and anti-β-actin (loading control) antibodies. *B,* cells were cultured in K-SFM in the presence or absence of 3% FBS for 3 days. Cell numbers were estimated by the sulforhodamine B assay. *Points,* means from three independent experiments done in quadruplicate; *bars*, SD. The concentrations of celecoxib shown are IC_{50} values calculated from the concentration-response curves of each cell line using DMSO as a control. *C,* expression of COX-2 in 1198 cells treated with celecoxib (2.5 and 5 μmol/L), 4HPR (0.25 μmol/L), and their combinations for 3 days. A549 cells were included as a positive control for COX-2. β-Actin served as the loading control.

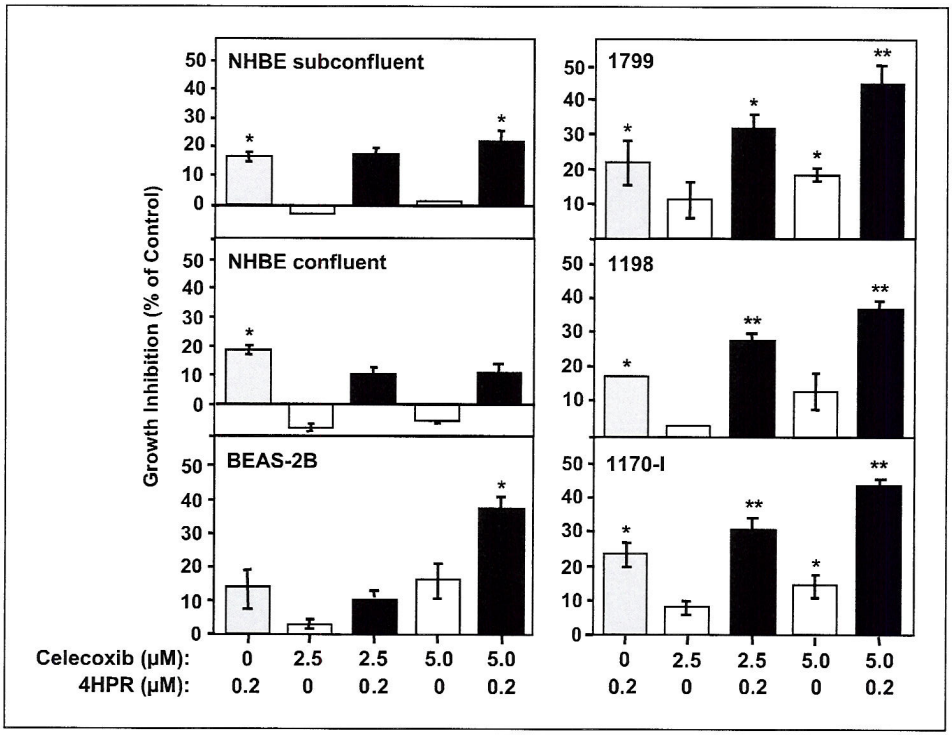


Figure 2. Comparison of the effects of celecoxib, 4HPR, and their combinations on the growth of human bronchial epithelial cell lines representing an *in vitro* model of lung carcinogenesis. Cells were seeded into 96-well culture plates and incubated overnight before treatment with celecoxib (2.5 and 5 μmol/L), 4HPR (0.2 μmol/L), or both agents for 3 days. Cultures incubated with DMSO alone served as a control. Cell numbers were estimated by the sulforhodamine B assay and analyzed for statistical significance using Student's paired *t* test with $P \leq 0.05$ (*) and $P \leq 0.005$ (**) related to control cultures in DMSO, respectively. *Columns*, means from three independent experiments done in quadruplicate; *bars*, SD.

agents, both 4HPR and celecoxib exerted some inhibitory effects on colony formation in all cell lines (Fig. 3). For example, 5 μmol/L celecoxib reduced the mean size of colonies in BEAS-2B cells as well as the size and number of colonies in the other cell lines. The colony-forming ability of 1799, 1198, and 1170-I cells was inhibited by 61%, 45%, and 27%, respectively ($P < 0.05$ for all comparisons), after treatment with 5 μmol/L celecoxib. 4HPR (0.2 μmol/L) was also effective by itself in reducing the size of colonies in BEAS-2B cells, however, without altering their number. On the contrary, 0.2 μmol/L 4HPR suppressed colony formation of 1799, 1198, and 1170-I cells by 36%, 15%, and 57%, respectively ($P < 0.05$, P = NS, and $P < 0.005$). 4HPR at the lower concentration of 0.1 μmol/L significantly inhibited the number of colonies in only 1198 and 1170-I cells by 24% and 38% ($P < 0.05$ and $P < 0.005$), respectively. Combining celecoxib and 4HPR substantially augmented the modest effect of treatment with the single agents. In particular, 5 μmol/L celecoxib plus 0.2 μmol/L 4HPR markedly suppressed colony formation in all cell lines by 86% (BEAS-2B) to 99% (1799, 1198, and 1170-I; $P < 0.005$ for all comparisons). Even 0.1 μmol/L 4HPR plus 5 μmol/L celecoxib significantly reduced the number of colonies in 1799, 1198, and 1170-I cells by 92%, 79%, and 63% ($P < 0.005$ for all comparisons), respectively, while decreasing the size of colonies in BEAS-2B cells without altering their number (Fig. 3).

Celecoxib plus 4HPR induces apoptosis in BEAS-2B and 1198 cells more efficiently than either agent alone. Treatment of BEAS-2B and 1198 cells with up to 5 μmol/L celecoxib had negligible effects on the induction of apoptosis as determined by the TUNEL assay (Fig. 4*A* and *B*). On the other hand, treatment with 0.25 μmol/L 4HPR increased the amount of apoptotic BEAS-2B and 1198 cells to 10 ± 2.4% and 18 ± 1.7%, respectively. However, combinations of 0.25 μmol/L 4HPR and celecoxib (≤5 μmol/L) significantly augmented apoptosis compared with treatment with either agent alone ($P \leq 0.05$ and $P \leq 0.005$ compared with untreated culture). For example, incubation with 5 μmol/L celecoxib plus 0.25 μmol/L 4HPR caused ~60% apoptotic BEAS-2B and 1198 cells, respectively.

Effects of celecoxib plus 4HPR on apoptosis-related proteins in BEAS and 1198 cells. The expression of proteins related to apoptosis was applied to confirm apoptotic events induced by the combination of celecoxib and 4HPR in BEAS-2B and 1198 cells. As a result, we obtained a decrease in the expression levels of procaspase-3 and procaspase-9, indicating caspase activation concomitant with an increase in cleavage of the 113 kDa PARP to the 89 kDa fragment (Fig. 4*C* and *D*). These effects were most profound in cells treated with a combination of 5 μmol/L celecoxib and 0.25 μmol/L 4HPR. Notably, in BEAS-2B cells, 4HPR alone diminished the level of procaspase-3 and increased the cleavage of PARP. No changes were detected in the expression levels of caspase-8 in either cell line. The level of the antiapoptotic protein Bcl-2 decreased and that of the proapoptotic molecule Bcl-X_S increased in both cell lines in response to combined treatment, whereas the expression of the proapoptotic protein Bax and the antiapoptotic protein Bcl-X_L was unaltered regardless of treatment. We further examined the effects of celecoxib and 4HPR on the mitochondrial pathway by analysis of cytochrome *c* release, which contributes to caspase-3 and caspase-9 activation, resulting in the degradation of PARP and subsequent cleavage of internucleosomal DNA. Treatment of 1198 cells with 5 μmol/L celecoxib plus 0.25 μmol/L 4HPR markedly induced cytochrome *c* release from the mitochondria into the cytosol (Fig. 4*D*, *bottom*). Altogether, these TUNEL and Western blot data confirm the apoptosis-inducing effects of combined celecoxib and 4HPR treatment.

Celecoxib plus 4HPR induces apoptosis partly by suppressing the Akt signaling pathway in 1799 cells. To determine whether combinations of celecoxib and 4HPR can alter the Akt signaling pathway in 1799 transformed human bronchial epithelial cells, we investigated the effects of these agents alone and in combination on the activation of Akt and its downstream molecule

glycogen synthase kinase-3β (GSK-3β). The constitutively high levels of phosphorylated Akt and phosphorylated GSK-3β were marginally decreased in response to 5 μmol/L celecoxib or 0.25 μmol/L 4HPR alone, but greatly decreased by the combination of these two agents. Likewise, the expression of the antiapoptotic protein Bcl-2 only declined after incubation with celecoxib in combination with 4HPR. No alterations appeared in the expression of Akt and GSK-3α/β after treatment with celecoxib and/or 4HPR compared with DMSO control cultures (Fig. 5*A* and *B*).

To further examine whether expression of constitutively active Akt can protect cells from undergoing apoptosis, we infected 1799 cells with adenoviral vector containing MyrAkt tagged with hemagglutinin for the recombinant protein (MyrAkt-HA) before treatment with celecoxib and 4HPR and compared the levels of apoptosis-related proteins in those cells with cells infected with adenoviral vector control (Ad5CMV). Successful infection is illustrated by the appearance of a single protein band by an antibody against HA-tag and a second band by an anti-Akt antibody (Fig. 5*C, top two panels*). As expected, these bands were not detected in cells infected with at Ad5CMV only. The 1799 cells expressing MyrAkt-HA were less sensitive than the cells infected with control vector to the apoptosis-inducing effects of celecoxib plus 4HPR as indicated by unaltered expressions of procaspase-3, Bcl-2, and decreased levels of PARP cleavage (Fig. 5*C*).

Discussion

Celecoxib has been shown to regress colorectal adenomas in patients with familial adenomatous polyposis relative to placebo, which resulted in the Food and Drug Administration approving celecoxib for adjunctive management of this disease (10). The presumed mechanism of action of celecoxib is selective inhibition of COX-2, an enzyme responsible for the metabolic conversion of arachidonic acid to prostaglandins, which play important roles in inflammation, cell proliferation, cell survival, and carcinogenesis (7). Moreover, the induction of COX-2 represents an early event in cancer development, and its expression seems to be associated with a poor prognosis in various types of cancer, including lung

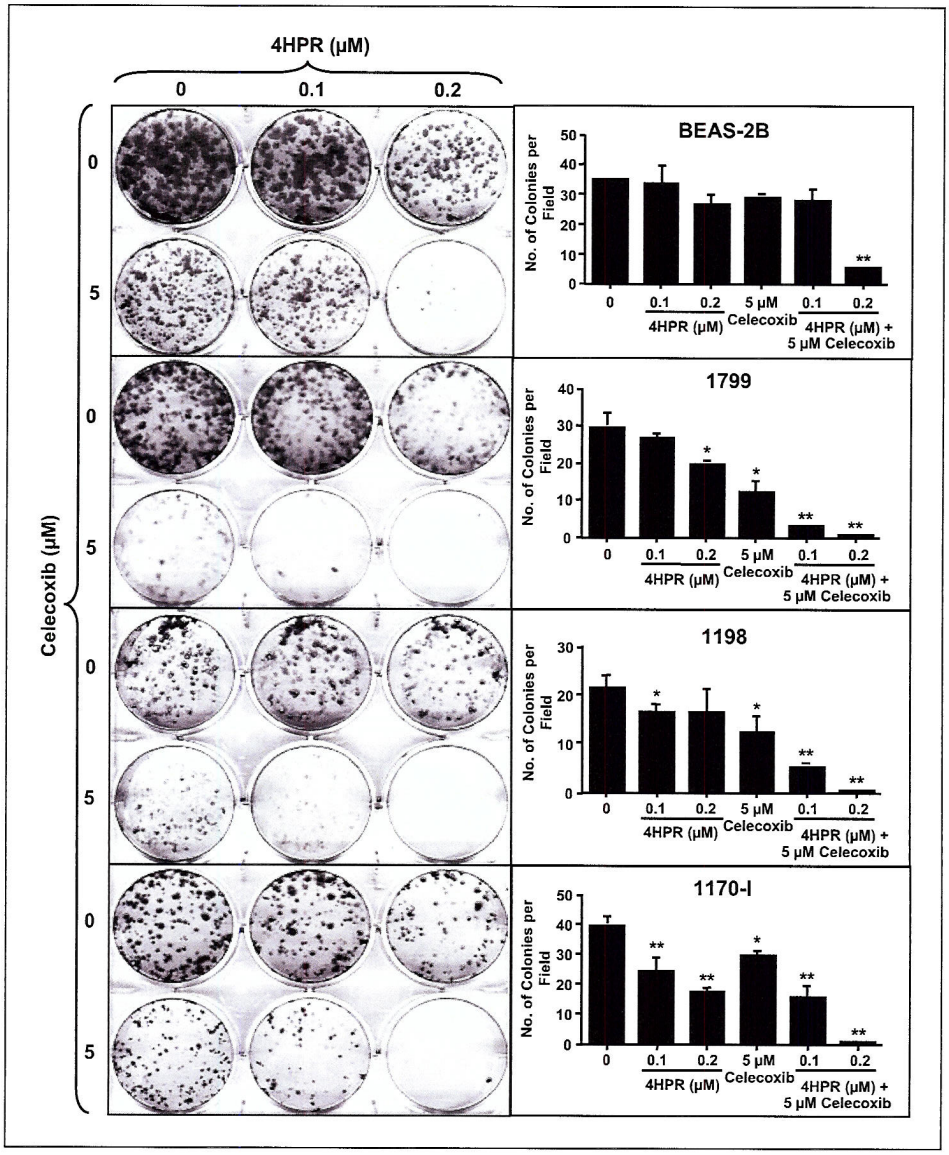


Figure 3. Effects of celecoxib, 4HPR, and their combinations on the colony-forming ability of human bronchial epithelial cell lines representing an *in vitro* model of lung carcinogenesis. Exponentially growing cells were seeded into six-well culture plates and treated with celecoxib (5 μmol/L), 4HPR (0.1 and 0.2 μmol/L), and their combinations for 14 days. *Columns,* number of colonies for each cell line calculated from quadruplicate determinations; *bars*, SD. $P \leq 0.05$ (*) and $P \leq 0.005$ (**) compared with cultures in DMSO (*leftmost column*) by Student's paired *t* test.

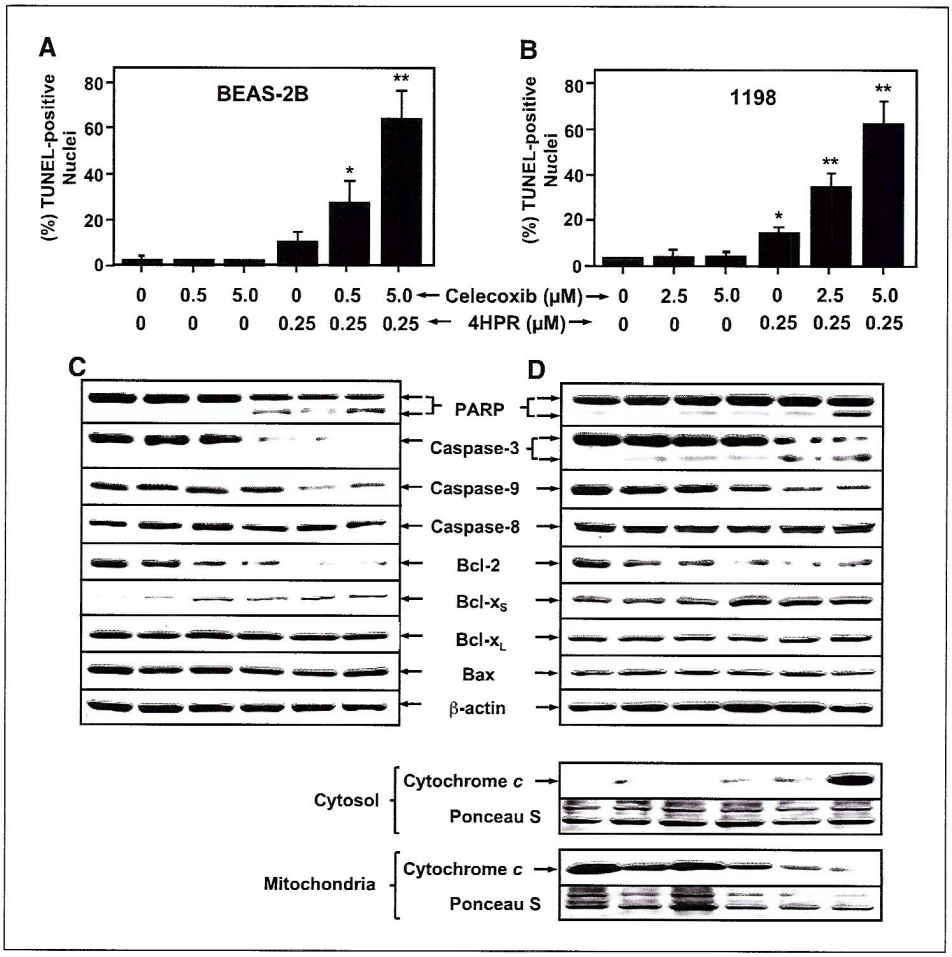


Figure 4. Effects of celecoxib, 4HPR, and their combinations on the induction of apoptosis and expression of apoptosis-related proteins in BEAS-2B and 1198 bronchial epithelial cell lines. *A* and *B*, the cells were treated with celecoxib (≤5 μmol/L), 4HPR (0.25 μmol/L), or both for 3 days before being harvested and analyzed by TUNEL assay. *Columns*, percentage of TUNEL-positive nuclei representative of two independent experiments and calculated for statistical significance using Student's two-tailed *t* test with $P \leq 0.05$ (*) and $P \leq 0.005$ (**), respectively. *C* and *D*, cells treated as above were harvested, lysed, and subjected to Western blot analysis using antibodies against the indicated proteins. The release of cytochrome *c* into the cytosol of 1198 cells was determined by Western blotting after fractionation of mitochondria and cytosol. β-Actin and Ponceau S staining were used as loading controls.

cancer (8, 9, 18). The fact that celecoxib at clinically achievable concentrations markedly decreased the PGE_2 production in COX-2–expressing cells supports its use as a cancer therapeutic and chemopreventive agent (17, 18, 20–24).

However, recent studies have suggested that the activity of celecoxib *in vivo* may not be exclusively mediated by a COX-2–dependent pathway (23, 24). For example, Sinicrope et al. (37) reported that celecoxib administered at 400 mg twice daily over 6 months induced polyp regression in patients with familial adenomatous polyposis by modulating *in vivo* rates of cell proliferation and apoptosis without evidences for suppression of PGE_2 in either normal tissue or adenomas. Furthermore, the concentrations of celecoxib needed to exert antitumor activity (e.g., apoptosis) *in vitro* are at least 10-fold higher than those required to inhibit COX-2 activity *in vivo*, raising the question whether the mechanisms identified *in vitro* are applicable to tumor responses *in vivo* (23–25). In addition, experimental studies have consistently shown that the sensitivity of various cancer cells to celecoxib is not related to their COX-2 expression status (24, 25).

We have used an *in vitro* model of human lung carcinogenesis to investigate the effects of clinically relevant concentrations of celecoxib in combination with the synthetic retinoid 4HPR. This model consists of distinct bronchial epithelial cell lines derived from SV40 large T-antigen immortalized BEAS-2B cells after exposure to cigarette smoke condensate and has proven useful in studying the process of carcinogenic transformation and the efficacy of cancer chemopreventive agents (30, 31). Our results indicate that cell lines constituting this model showed no detectable COX-2 expression, had corresponding low levels of PGE_2, and were nonetheless sensitive to the growth-inhibitory activity of celecoxib, such as A549 cancer cells, which constitutively expressed COX-2. Because the IC_{50} values for celecoxib were higher than those achievable in clinical trials (23), we decided to combine celecoxib with the proapoptotic retinoid 4HPR. Our data show that treatment of premalignant and tumorigenic cell lines with celecoxib or 4HPR at clinically relevant concentrations exhibited only modest inhibitory effects (<20%) on cell growth. However, incubation of these cell lines with 5 μmol/L celecoxib plus 0.2 μmol/L 4HPR revealed additive effects on growth suppression in monolayer cultures determined after 3 days of incubation.

The preclinical evaluation of therapeutic and chemopreventive drugs typically involves the comparison of the effects on premalignant and normal cells to determine whether a "therapeutic window" exists. Clearly, chemopreventive agents are required to have a better safety profile than therapeutic drugs because they may be administered over a prolonged period of time. Both celecoxib (10, 37) and 4HPR (29, 38) have been found to have low or no side effects in humans at doses that lead to plasma levels comparable with concentrations we used in this study and even considerably higher in the case of 4HPR (38). Therefore, we anticipated finding low or no effects of these agents on NHBE cells. However, celecoxib inhibited the growth, especially of subconfluent

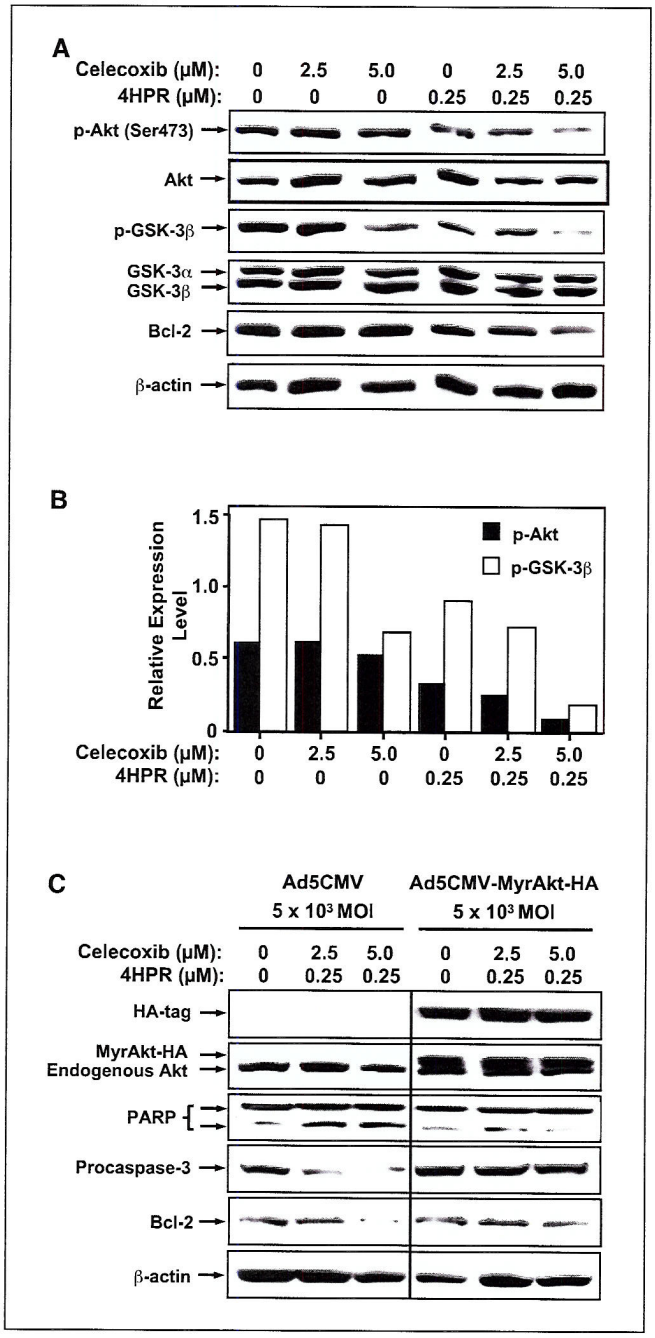


Figure 5. Effects of celecoxib, 4HPR, and their combinations on the Akt survival pathway in 1799 human transformed bronchial epithelial cells. *A*, after 2 days of treatment with celecoxib and 4HPR, protein extracts (50 μg/lane) prepared from the cells were subjected to Western blot analysis to determine the expression of phosphorylated Akt (Ser^{473}), phosphorylated GSK-3β, Akt, GSK-3α/β, and Bcl-2. *B*, the blots were scanned using SF Launcher v2.0.5, and the densities of p-Akt (Ser^{473}) and p-GSK-3β were quantified in relation to the corresponding total protein expression and normalized to β-actin by NIH image 1.58 software. *C*, effects of constitutively active Akt on the response of 1799 bronchial epithelial cells to apoptosis induced by celecoxib and 4HPR. The cells were infected at 5×10^3 MOI of adenoviral vector control (Ad5CMV) or adenoviral vector containing myristoylated Akt (Ad5CMV-MyrAkt-HA) in keratinocyte SFM for 1 day before incubation with celecoxib/4HPR for an additional 2 days. Successful infection is illustrated by the appearance of a single protein band by an antibody against HA-tag and a second band by an anti-Akt antibody (*top two panels*). As expected, these bands were not detected in cells infected with at Ad5CMV. Apoptosis-inducing activity was determined by the expression of procaspase-3, Bcl-2, and cleavage of PARP. β-Actin served as a control for protein loading.

proliferating NHBE cultures, albeit with lower potency than it did inhibit premalignant and tumorigenic cell proliferation. Interestingly, at low concentrations (2.5 and 5 μmol/L), celecoxib failed to inhibit NHBE cell growth, whereas it suppressed the growth of their premalignant and tumorigenic counterparts. 4HPR (0.2 μmol/L) showed significant but low inhibition compared with untreated cultures; however, combinations with celecoxib did not augment this effect. Thus, these agents appear to exert a selective effect on premalignant and tumorigenic cells compared with NHBE cells. Notably, the use of NHBE cells as a control is not straightforward because these cells are cultured under conditions where they are stimulated to proliferate, i.e., K-SFM supplemented with epidermal growth factor and bovine pituitary extract, hence emulate hyperplasia rather than normal bronchial epithelium. In fact, the proliferative index of histologically normal lung epithelium from nonsmokers *in vivo* is 0.16 ± 0.15% and 1.26 ± 1.17% in biopsies obtained from smokers (39) as opposed to 4.8% to 36% in premalignant lesions such as dysplasia (39, 40). Therefore, we investigated the effects of the agents on both subconfluent (proliferating) and confluent (quiescent) NHBE cells. Indeed, we found that confluent NHBE cells were less inhibited by high concentrations of celecoxib or low concentrations of celecoxib combined with 4HPR than premalignant and tumorigenic cells.

Combinations of low doses of both celecoxib and 4HPR resulted in a more dramatic inhibitory activity on the formation of colonies, where cells were seeded at low density and treated for 14 days, compared with growth inhibition in cultures at higher density measured after 3 days of incubation. We observed that BEAS-2B cells exhibited lower sensitivity to combinations of celecoxib and 4HPR than 1799, 1198, and 1170-I cells did (Figs. 2 and 3). The reason for such difference is unclear. However, it is noteworthy that the three more sensitive cell lines were derived from BEAS-2B cells after growing as xenotransplants in nude mice for 6 months (30). This *in vivo* passage may have been accompanied by selective pressure that resulted in some distinction from the parental cell line, which was immortalized with SV40 large T-antigen. Recently, we found that various human bronchial epithelial cell lines immortalized with hTERT and CDK4 (41) also lack COX-2 expression yet exhibit higher sensitivity to the combination of celecoxib and 4HPR than to either agent alone (data not shown). Thus, we conclude that the sensitivity of premalignant bronchial epithelial cell lines to this combination is not restricted to SV40 large T-antigen immortalized cells.

To gain insight into the mechanisms by which celecoxib in combination with 4HPR enhanced the apoptosis of human bronchial epithelial cell lines, as observed with the TUNEL assay, we examined the expression levels of several apoptosis-related proteins. Our data indicate that celecoxib plus 4HPR activates the mitochondrial apoptosis pathway as evidenced by suppression of the antiapoptotic protein Bcl-2, increase in the proapoptotic protein Bcl-x_S, release of apoptogenic cytochrome *c* into the cytosol, activation of caspase-9 and caspase-3, and cleavage of PARP. However, no changes were detected in the expression levels of Bcl-x_L, Bax, or caspase-8. On the basis of our initial findings that none of these cell lines expressed COX-2, we concluded that the growth-inhibitory and apoptosis-inducing effects of celecoxib in combination with 4HPR are mediated by COX-2–independent mechanisms. Numerous studies have shown that celecoxib induces apoptosis in a variety of cell types, but only at concentrations between 50 and 100 μmol/L (23, 25, 42–44). The mechanism ascribed to these effects was based on activation of the

mitochondrial signaling pathway, as indicated by breakdown of the mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of PARP via a Bcl-2/Bcl-x_L–independent pathway in rat cholangiocarcinoma (42), human lymphoma (43), and prostate carcinoma (44) cell lines. Correspondingly, various reports have delineated the proapoptotic effects of 4HPR at concentrations above 3 μmol/L by a mechanism that includes augmented generation of reactive oxygen species from the mitochondria with subsequent release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of PARP without altering Bcl-2 and Bax (25–27). However, ectopic overexpression of Bcl-2 did not protect T-cell acute lymphoblastic leukemia cells from apoptosis induced by 4HPR but markedly delayed its onset (45). Nonetheless, in those cells, 4HPR induced apoptosis via a mitochondrial (reactive oxygen species mediated) pathway that involves the obligatory contributions of the proapopotic Bcl-2 family members Bax and/or Bak (46).

It is well established that alterations to the Akt signaling pathway are frequent in human malignancies that result in enhanced resistance to apoptosis through multiple mechanisms (47). In fact, previous studies on rat cholangiocarcinoma (42), human prostate (44), hepatocellular (48), and colon cancer (49) cells have shown that celecoxib inhibits Akt phosphorylation in association with induction of apoptosis via the mitochondrial pathway. However, these effects required high concentrations of celecoxib that are not attainable *in vivo*. In contrast, our data indicate that incubation of premalignant bronchial epithelial cells with clinically relevant concentrations of celecoxib combined with 4HPR augments apoptosis by reducing phosphorylation levels of Akt and its direct downstream substrate GSK-3β. This conclusion was supported by further experiments showing that overexpression of constitutive active Akt protects, at least partly, premalignant cells from undergoing apoptosis induced by celecoxib plus 4HPR. Taken together, our findings strongly warrant additional evaluation of the efficacy of this combination in chemoprevention and therapy of lung cancer in animal models and eventually in clinical trials.

Acknowledgments

Received 11/17/2005; revised 7/24/2006; accepted 8/7/2006.

Grant support: National Cancer Institute grant PO1 CA98144; Department of Defense Biology, Education, Screening, Chemoprevention, and Treatment Lung Cancer Program grant DAMD17-01-1-0689-2; Cancer Center Support grant P30 CA 16672; and the David Workman Memorial Endowment Award from the Samuel Waxman Cancer Research Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. James Zweibel (National Cancer Institute, Bethesda, MD) for providing 4HPR, Wendy Schober (Department of Blood and Marrow Transplantation, University of Texas M.D. Anderson Cancer, Houston, TX) for her assistance in the acquisition of flow cytometry data, and Peiying Yang and Robert A. Newman (Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer) for the PGE_2 analysis.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10–30.
2. Cohen V, Khuri FR. Chemoprevention of lung cancer: concepts and strategies. Expert Rev Anticancer Ther 2005;5:549–65.
3. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science 1997;278:1073–7.
4. Muscat JE, Chen SQ, Richie JP, Jr., et al. Risk of lung carcinoma among users of nonsteroidal antiinflammatory drugs. Cancer 2003;97:1732–6.
5. Hirsch FR, Lippman SM. Advances in the biology of lung cancer chemoprevention. J Clin Oncol 2005;23:3186–97.
6. Rao CV, Reddy BS. NSAIDs and chemoprevention. Curr Cancer Drug Targets 2004;4:29–42.
7. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999;18:7908–16.
8. Khuri FR, Wu H, Lee JJ, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861–7.
9. Hida T, Yatabe Y, Achiwa H, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 1998;58:3761–4.
10. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000;342:1946–52.
11. Bardou M, Barkun AN, Ghosn J, Hudson M, Rahme E. Effect of chronic intake of NSAIDs and cyclooxygenase 2-selective inhibitors on esophageal cancer incidence. Clin Gastroenterol Hepatol 2004;2:880–7.
12. Reddy BS, Hirose Y, Lubet RA, Steele VE, Kelloff GJ, Rao CV. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res 2000;60:293–7.
13. Hu PJ, Yu J, Zeng ZR, et al. Chemoprevention of gastric cancer by celecoxib in rats. Gut 2004;53:195–200.
14. Diperna CA, Bart RD, Sievers EM, Ma Y, Starnes VA, Bremner RM. Cyclooxygenase-2 inhibition decreases primary and metastatic tumor burden in a murine model of orthotopic lung adenocarcinoma. J Thorac Cardiovasc Surg 2003;126:1129–33.
15. Klenke FM, Gebhard MM, Ewerbeck V, Abdollahi A, Huber PE, Sckell A. The selective Cox-2 inhibitor Celecoxib suppresses angiogenesis and growth of secondary bone tumors: an intravital microscopy study in mice. BMC Cancer 2006;12:6–9.
16. Harris RE, Alshafie GA, Abou-Issa H, Seibert K. Chemoprevention of breast cancer in rats by celecoxib, a cyclooxygenase-2 inhibitor. Cancer Res 2000;60:2101–3.
17. Li N, Sood S, Wang S, et al. Overexpression of 5-lipoxygenase and cyclooxygenase 2 in hamster and human oral cancer and chemopreventive effects of zileuton and celecoxib. Clin Cancer Res 2005;11:2089–96.
18. Gupta S, Adhami VM, Subbarayan M, et al. Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. Cancer Res 2004;64:3334–43.
19. Grubbs CJ, Lubet RA, Koki AT, et al. Celecoxib inhibits *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. Cancer Res 2000;60:5599–602.
20. Fischer SM, Lo HH, Gordon GB, et al. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, and indomethacin against ultraviolet light-induced skin carcinogenesis. Mol Carcinog 1999;25:231–40.
21. Mao JT, Cui X, Reckamp K, et al. Chemoprevention strategies with cyclooxygenase-2 inhibitors for lung cancer. Clin Lung Cancer 2005;7:30–9.
22. Abou-Issa H, Alshafie G. Celecoxib: a novel treatment for lung cancer. Expert Rev Anticancer Ther 2004;4:725–34.
23. Niederberger E, Tegeder I, Vetter G, et al. Celecoxib loses its anti-inflammatory efficacy at high doses through activation of NF-κB. FASEB J 2001;15:1622–4.
24. Schroeder CP, Yang P, Newman RA, Lotan R. Eicosanoid metabolism in squamous cell carcinoma cell lines derived from primary and metastatic head and neck cancer and its modulation by celecoxib. Cancer Biol Ther 2004;3:847–52.
25. Sun SY, Schroeder CP, Yue P, Lotan D, Hong WK, Lotan R. Enhanced growth inhibition and apoptosis induction in NSCLC cell lines by combination of celecoxib and 4HPR at clinically relevant concentrations. Cancer Biol Ther 2005;4:407–13.
26. Suzuki S, Higuchi M, Proske RJ, Oridate N, Hong WK, Lotan R. Implication of mitochondria-derived reactive oxygen species, cytochrome *c* and caspase-3 in *N*-(4 hydroxyphenyl)retinamide-induced apoptosis in cervical carcinoma cells. Oncogene 1999;18:6380–7.
27. Zou CP, Kurie JM, Lotan D, Zou CC, Hong WK, Lotan R. Higher potency of *N*-(4-hydroxyphenyl)retinamide than all-*trans*-retinoic acid in induction of apoptosis in non-small cell lung cancer cell lines. Clin Cancer Res 1998;4:1345–55.
28. Sun SY, Hail N, Jr., Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 2004;96:662–72.
29. Guerrieri-Gonzaga A, Robertson C, Bonanni B, et al. Preliminary results on safety and activity of a randomized, double-blind, 2 × 2 trial of low-dose tamoxifen and fenretinide for breast cancer prevention in premenopausal women. J Clin Oncol 2006;24:129–35.
30. Klein-Szanto AJ, Iizasa T, Momiki S, et al. A tobacco-specific *N*-nitrosamine or cigarette smoke condensate causes neoplastic transformation of xenotransplanted human bronchial epithelial cells. Proc Natl Acad Sci U S A 1992;89:6693–7.
31. Reddel RR, Ke Y, Gerwin BI, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 1988;48:1904–9.
32. Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.
33. Li X, Traganos F, Melamed MR, Darzynkiewicz Z. Single-step procedure for labeling DNA strand breaks with fluorescein-or BODIPY-conjugated deoxynucleotides: detection of apoptosis and bromodeoxyuridine incorporation. Cytometry 1995;20:172–80.
34. Chun KH, Kosmeder JW, Sun S, et al. Chemopreventive effects of deguelin, a naturally occurring PI3K/Akt inhibitor, during the malignant transformation of human bronchial epithelial cells. J Natl Cancer Inst 2003;95:291–302.

35. Ji L, Nishizaki M, Gao B, Burbee D, et al. Expression of several genes in the human chromosome 3p21.3 homozygous deletion region by an adenovirus vector results in tumor suppressor activities *in vitro* and *in vivo*. Cancer Res 2002; 62:2715–20.
36. Franke TF, Yang SI, Chan TO, et al. The protein kinase encoded by the Akt protooncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 1995;81:727–36.
37. Sinicrope FA, Half E, Morris JS, et al. Cell proliferation and apoptotic indices predict adenoma regression in a placebo-controlled trial of celecoxib in familial adenomatous polyposis patients. Cancer Epidemiol Biomarkers Prev 2004;13:920–7.
38. Garaventa A, Luksch R, Lo Piccolo MS, et al. Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. Clin Cancer Res 2003;9: 2032–9.
39. Hiroshima K, Iyoda A, Shibuya K, et al. Evidence of neoangiogenesis and an increase in the number of proliferating cells within the bronchial epithelium of smokers. Cancer 2002;95:1539–45.
40. Lantuejoul S, Soria JC, Morat L, et al. Telomere shortening and telomerase reverse transcriptase expression in preinvasive bronchial lesions. Clin Cancer Res 2005;11:2074–82.
41. Ramirez RD, Sheridan S, Girard L, et al. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Cancer Res 2004;64:9027–34.
42. Zhang Z, Lai GH, Sirica AE. Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. Hepatology 2004;39:1028–37.
43. Jendrossek V, Handrick R, Belka C. Celecoxib activates a novel mitochondrial apoptosis signaling pathway. FASEB J 2003;17:1547–9.
44. Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. J Biol Chem 2000;275:11397–403.
45. Delia D, Aiello A, Formelli F, et al. Regulation of apoptosis induced by the retinoid *N*-(4-hydroxyphenyl) retinamide and effect of deregulated bcl-2. Blood 1995; 85:359–67.
46. Boya P, Morales MC, Gonzalez-Polo RA, et al. The chemopreventive agent *N*-(4-hydroxyphenyl) retinamide induces apoptosis through a mitochondrial pathway regulated by proteins from the Bcl-2 family. Oncogene 2003;22:6220–30.
47. Thompson JE, Thompson CB. Putting the rap on Akt. J Clin Oncol 2004;22:4217–26.
48. Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. Hepatology 2003;38:756–68.
49. Arico S, Pattingre S, Bauvy C, et al. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. J Biol Chem 2002;277: 27613–21.

Simultaneous inhibition of COX-2 and 5-LOX activities augments growth arrest and death of premalignant and malignant human lung cell lines

Claudia P. Schroeder[1], Peiying Yang[2], Robert A. Newman[2], Reuben Lotan[1]

[1] Department of Thoracic, Head and Neck Medical Oncology, and [2] Department of Experimental Therapeutics; The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

Correspondence to: Reuben Lotan, Ph.D., MDACC, 1515 Holcombe Boulevard Box 432, Houston, TX 77030 USA. Telephone: +1.713.792.8467. Fax: +1.713.745.5656. E-mail: rlotan@mdanderson.org

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Cancer Institute (PO1 CA98144), and the BESCT Lung Cancer Program from the Department of Defense (DAMD17-01-1-0689-2).

ABBREVIATED TITLE

Combination of COX-2 and 5-LOX inhibitors on human lung cell lines

ABSTRACT

The arachidonic acid-metabolizing enzymes cyclooxygenase-2 (COX-2) or 5-lipoxygenase (5-LOX) are overexpressed during lung carcinogenesis and their end products (e.g.; PGE_2, 5-HETE, and LTB_4) have been implicated in tumor development. Recently, COX-2 inhibitors (e.g.; celecoxib) and 5-LOX inhibitors (e.g.; MK886 and REV5901) used as single agents have shown promising activities in the treatment and chemoprevention of cancer. However, little is known about the effects of combinations of these inhibitors. We found that simultaneous treatment of premalignant and malignant human lung cell lines with celecoxib, MK886, and REV5901 is more potent in growth suppression and induction of cell death than single or dual combination of these agents. However, their sensitivity to the inhibitors was not directly associated with the expression of COX-2, 5-LOX, or 5-LOX-activating protein (FLAP), but correlated with the production of corresponding metabolites. Furthermore, partial protection of cell death was observed when PGE_2 and/or 5-HETE was added to cell cultures treated with celecoxib, MK886, and REV5901 simultaneously. Our data indicate that a triple drug combination of distinct inhibitors of the eicosanoid metabolism at clinically feasible concentrations were more effective than each agent alone suggesting further investigations.

Key words: COX-2, 5-LOX, MK886, REV5901, celecoxib, human lung cell lines

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the United States with a less than 15% five-year survival rate, which is only a small improvement over the past several decades[1]. Therefore, the discovery of new strategies and potential agents in order to control the development and progression of human lung cancer is urgently needed.

It has been known that a higher rate of the eicosanoid metabolism, involving the breakdown of arachidonic acid (AA) to multiple endproducts by cyclooxygenases (COXs) and lipoxygenases (LOXs), plays a role in most human epithelial cancers including lung cancer[2-4]. For instance, inducible COX-2 is a known indicator for pathological conditions such as inflammation and cancer, especially those of non-small cell lung cancer histology, which has also been linked to a poorer prognosis[5-7]. Moreover, COX-2 is frequently upregulated in preneoplastic lesions or atypical bronchiolar metaplasia compared with normal lung[8]. As a result, the excessive production of COX-2-derived metabolites, mainly prostaglandin E_2 (PGE_2), stimulates proliferation, reduces apoptosis, and promotes angiogenesis[9,10]. Likewise, abnormalities in the 5-LOX pathway occur frequently during neoplastic transformation of lung tissue involving over-expression of 5-LOX and increased levels of its products, 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4 (LT)B_4, that have been associated with proliferative, anti-apoptotic, and angiogenic properties[9,11,12]. Consequently, inhibition of COX-2 and 5-LOX activities suppressed growth and induced apoptosis in a variety of cancer cell lines and preclinical models[13-21]. Moreover, celecoxib and various 5-LOX inhibitors (e.g.; MK886) prevented lung tumorigenesis in carcinogen-induced mouse models[2,15,16]. Interestingly, it has been

reported that celecoxib at clinically feasible concentrations not only inhibits the synthesis of PGE_2 but is also capable of modulating various metabolites generated from the LOX pathway, especially 5-HETE and LTB_4 metabolites which derive from the 5-LOX cascade[18,22,23]. Because a dynamic transition between the COX and LOX pathway may play a role in tumor development and progression, it is plausible to target both enzymatic pathways by using COX in combination with LOX inhibitors[20,23,24].

The present study examines the effects of the selective COX-2 inhibitor celecoxib in combination with the selective 5-LOX inhibitor REV5901, and the 5-LOX-activating protein (FLAP) inhibitor MK886 on suppression of cell growth of premalignant and malignant human lung cell lines.

MATERIAL AND METHODS

Reagents and materials

Dulbecco's modified eagle's minimal essential medium (DMEM), keratinocyte serum-free medium (SFM), phosphate-buffered saline (PBS), and trypsin were purchased from Gibco™ Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum (FBS) was from HyClone Laboratories, Inc. (Logan, UT). The COX-2-inhibitor celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) was obtained from GD Searle & Co (Chicago, IL), the FLAP-inhibitor MK866 (1-[(4-chlorophenzyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl-1H-indole-2-propanoic acid, sodium salt) was from Biomol Research Laboratories (Plymouth Meeting, PA) and the 5-LOX-inhibitor REV5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol) was purchased from Cayman Chemicals Co (Ann Arbor, MI). Bovine serum albumin, dimethyl sulfoxide (DMSO), ethylene diamine tetra acetic acid, and sodium dodecyl sulfate (SDS) were from Sigma Chemical Co. (St. Louis, MO). All culture plastic ware was purchased from BD Bioscience Labware (Bedford, MA). The deuterium-labeled eicosanoids PGE_2, LTB_4, and 5-HETE used as internal standard for ESI-LC/MS/MS analyses were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Cell culture

The cell lines used in this study represent an in vitro model of human lung carcinogenesis including BEAS-2B, bronchial epithelial cells immortalized using SV40/adenovirus-12 hybrid T-antigen, and transformed (1198) or tumorigenic (1170-I)

cells derived from BEAS-2B by exposure to cigarette smoke condensate (CSC) in vivo after transplantation into nude mice[25]. Immortalized 1799 cells were derived from BEAS-2B by a similar in vivo transplantation without exposure to CSC[26]. These cell lines were obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). The non-small cell lung cancer cell lines A549 was purchased from the American Type Cell Culture Collection (ATCC; Rockville, MD). BEAS-2B and 1799 cells were grown in keratinocyte-SFM supplemented with EGF (2.5 µg) and bovine pituitary extract (25 µg) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cell lines 1198 and 1170-I were maintained in keratinocyte-SFM containing 3% FBS and bovine pituitary extract (25 µg). A549 cells were cultured in a mixture of DMEM/Ham's F12 medium (1:1, v/v) supplemented with 5% FBS.

Cell growth studies, apoptosis

After trypsinization, 6 x 10^3 cells/well were seeded into 96-well culture plates, allowed to adhere overnight prior to treatment with celecoxib, MK886, REV5901, and their combinations at different concentrations for 72 hrs. Control cultures were grown in medium containing DMSO (0.02%, v/v). Cell number was determined by the sulforhodamine B (SRB) assay using an automated plate reader MR5000 (Dynatech Laboratories Inc., Chantilly, VA)[27]. The inhibition of cell growth was calculated from the equation, % inhibition = (1-OD*t*/OD*c*) x 100%, whereas OD*t* and OD*c* represent optical densities of treated and control cultures, respectively. Concentration response curves were plotted and concentrations of the agents used in this study resulting in 50% growth inhibition (IC_{50}) were calculated by interpolation. The extent of apoptotic and necrotic cells was determined by double staining with Annexin V-FITC and propidium iodide

using Annexin-V-FLUOS staining kit from Roche Applied Science (Indianapolis, IN). The cells were seeded into 100 x 20 mm tissue culture dish and allowed to adhere overnight before incubation with the COX and LOX inhibitors. Control cultures were treated with medium containing solvent vehicle only (0.02%, v/v DMSO). After 72 hrs, detached and adherent cells were collected, washed, and stained according to the manufacturer's instructions. Data analysis was performed on a FACSCalibur® cytometer utilizing the CellQuest™ software (Becton Dickinson, San Jose, CA). Three independent experiments were conducted.

Western blot analysis

Samples containing 50 µg of total cellular protein mixed in sample buffer (0.5 M Tris, pH 6.8; 0.3% glycerol; 0.03% β-mercaptoethanol; 10% SDS; 0.001% bromphenolblue) were electrophoretically separated through 10% SDS-polyacrylamide slab gels followed by transfer onto Trans-Blot® nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Briefly, cell monolayers were washed twice with ice-cold PBS and collected in lysis buffer containing 150 mM NaCl; 0.02% NaN_3; 2% Igepal CA-630; 0.5% sodium deoxycholate; 0.2% SDS, and 50 mM Tris-HCl, pH 8.0 supplemented with protease inhibitors leupeptin (1 µg/ml), aprotinin (1 µg/ml), pepstatin (0.5 µg/ml), and phenylmethylsulfonyl fluoride (100 µg/ml). Protein concentrations were measured using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). After blocking with 3% nonfat dry milk solution in 0.1% (w/v) Tween 20 in PBS, the membranes were probed with anti-human antibodies at appropriate dilutions against COX-2 (Oxford Biomedical Research Inc., Oxford, MI), 5-LOX (BD Transduction Lab, Lexington, KY), and FLAP (Santa Cruz Biotechnology Inc., CA). Antibody binding was detected with horseradish

peroxidase-linked second antibody and enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). Loading and transferring control was confirmed by probing the membranes with anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO).

Measurement of PGE_2, 5-HETE, and LTB_4

Electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC/MS/MS) was performed to quantify eicosanoid metabolites as described elsewhere[28,29]. Briefly, an aliquot of 10 μl of 10% BHT and 10 μl of a mixture of internal standards (PGE_2-d_4, 5-HETE-d_8, LTB_4) was added to 1 ml of culture medium. Eicosanoids were eluted with 1 ml of methanol and evaporated under a stream of nitrogen. Samples were reconstituted in 100 μl methanol: 10 mM ammonium acetate buffer (v/v, 70:30, pH 8.5) prior to analysis. ESI-LC/MS/MS was performed using a Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA) equipped with an Agilent HP1100 binary pump HPLC inlet. Metabolites were separated using a Luna 3 μm phenyl hexyl 2 x 150 mm analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate (pH 8.5) and methanol. The flow rate was 250 μl/min with a column temperature maintained at 50°C and an injection volume of 25 μl. Fragmentation of all compounds was performed using argon as the collision gas at a collision cell pressure of 2.1×10^{-3} Torr. The results were expressed as nanograms of each eicosanoid per 10^6 cells. To normalize data, the cell number was measured with an electronic particle counter (Coulter, Hialeah, FL). Results shown represent mean values of at least two independent experiments.

Statistics

The results on growth inhibition induced by combinations of COX-2 and 5-LOX inhibitors represent mean values ± standard deviation (SD) of three independent experiments each performed in quadruplicates. Significance of difference between samples related to control cultures was determined using Student's paired *t*-test with probability (*P*) values less than 0.05 regarded as significant.

RESULTS

Production of PGE_2, 5-HETE, and LTB_4 and expression of the corresponding AA-metabolizing enzymes in premalignant and malignant human lung cell lines

To determine whether the AA metabolism is altered in cell lines representing an in vitro model of human lung carcinogenesis, we analyzed their production of PGE_2, 5-HETE, and LTB_4 using ESI-LC/MS/MS[28,29]. The endogenous PGE_2 levels in BEAS-2B and 1198 cell lines were very low (0.2 and 0.04 ng/10^6 cells, respectively), whereas A549 cancer cells produced 2.7 ng PGE_2/10^6 cells (Figure 1A). The synthesis of the 5-LOX metabolites 5-HETE and LTB_4 was low in BEAS-2B cells but higher and to a comparable amount in 1198 and A549 cell lines. We then determined the expression levels of COX-2, FLAP, and 5-LOX using immunoblotting. Figure 1B indicates that the enzyme expression showed a correlation with the production of the corresponding COX-2 and 5-LOX metabolites. For example, COX-2 was undetectable in premalignant bronchial epithelial cell lines, however, was strongly expressed in A549 cancer cells. FLAP was detected in all premalignant cell lines with a lower expression in BEAS-2B and a higher level in A549 cells. 5-LOX was expressed in 1198 and A549 cells compared to a faint detection in BEAS-2B, 1799, and 1170-I cell lines.

Effect of celecoxib, MK886, and REV5901 on growth arrest of premalignant and malignant human lung cell lines

Because the production of PGE_2, 5-HETE, and LTB_4 correlated with the expression levels of the corresponding enzymes, we asked whether the cell lines used in this study exhibit differential sensitivities to the selective COX-2 inhibitor celecoxib, the selective 5-LOX-inhibitor REV5901 and the FLAP-inhibitor MK886 (chemical structures, Figure 2). We found a dose-dependent growth inhibition after 72 hrs of incubation with IC_{50} values ranging from 15.9 to 30.4 μM for celecoxib, 0.3 to 1.7 μM for MK886, and 6.6 to 14.4 μM for REV5901 (Figure 3). Premalignant bronchial epithelial cell lines were more sensitive to the growth-inhibitory effects of celecoxib, MK886, or REV590 than A549 cancer cells as indicative as 1.5 to 2-fold lower IC_{50} values. Furthermore, MK886 that indirectly inhibits 5-LOX activity by blocking FLAP, was approximately 10-fold more potent on a molar basis compared to the selective enzyme inhibitors celecoxib or REV5901.

Simultaneous treatment with celecoxib, MK886, and REV5901 augments growth inhibition compared to treatment with one or two agents

Based on previous studies, which suggest a fine balance between the COX and LOX pathway in tumor cells, we hypothesized that simultaneous inhibition of the AA metabolism by using COX-2 in combination with 5-LOX inhibitors is more effective in suppression of cell growth than each of the inhibitors alone. Therefore, we treated 1198 premalignant and A549 malignant lung cell lines with pharmacologically achievable concentrations of celecoxib (5 μM), MK886 (1 μM), and REV5901 (5 μM) used as single agents and in combinations for 72 hrs before determination of cell numbers by the SRB method. Figure 4 shows that simultaneous treatment of the cells with all three inhibitors was more effective than incubation of the compounds alone or in dual combination. For

example, incubation of 1198 and A549 cells with a combination of celecoxib, MK886, and REV5901 suppressed their growth by 58% and 43%, respectively compared to untreated cultures ($P<0.05$). Besides, dual inhibition of COX-2 and 5-LOX enzymes through celecoxib and REV5901 induced a significant growth arrest by 29% and 37% in 1198 and A549 cell lines, respectively ($P<0.05$).

Cell death is increased by combinations of COX-2 and 5-LOX inhibitors and is partially prevented by PGE_2 and 5-HETE

To determine whether induction of apoptosis contributes to the growth-inhibitory activity of COX-2 and 5-LOX inhibitors, we performed double staining of the cells with Annexin V-FITC and propidium iodide followed by flow cytometric analysis. Figure 5 indicates that incubation of 1198 premalignant and A549 malignant cell lines with a triple drug combination of celecoxib (5 μM), MK886 (1 μM), and REV5901 (5 and 7.5 μM, respectively) induced 74.4% and 23.7% cell death, including apoptotic and necrotic cellular fraction, respectively. Addition of exogenous PGE_2 (0.5 μg/ml) and/or 5-HETE (100 nM) to cultures treated with the triple drug combination partially reduced the number of dead cells. For example, 1198 cell death induced by a combination of 5 μM celecoxib, 1 μM MK886, and 5 μM REV5901 was partially protected by 56% in the presence of 100 nM 5-HETE (Figure 5A). Moreover, A549 growth arrest was protected by 33% (from 23.7% to 7.9%) when both, PGE_2 and 5-HETE, were added to a combination of 5 μM celecoxib, 1 μM MK886, and 7.5 μM REV5901 (Figure 5B).

DISCUSSION

The deregulation of the AA metabolism in epithelial cancers including lung tumors represents an early sign of malignant transformation[3,9,10]. Hence, its metabolizing enzymes and end products provide promising targets for lung cancer chemoprevention and/or therapy. Indeed, inhibitors of the COX and LOX pathway have demonstrated anti-tumor and chemopreventive activity in lung cancer cells[13-16,21,22]. However, preclinical and clinical studies that have targeted just one metabolic pathway by using for instance only a COX-2 inhibitor (e.g.; celecoxib) showed modest impact[2,18,30]. It has also been suggested that blocking one metabolic cascade can lead to an enhancement of products generated from another cascade by re-directing the breakdown of the substrate AA. Specifically, others and we have demonstrated that suppression of PGE_2 by celecoxib at clinically relevant concentrations was accompanied by an increase of multiple 5-LOX metabolites, most significantly 5-HETE or LTB_4[18,22-24]. Therefore, it was of considerable interest to investigate whether a combination of inhibitors of the COX-2 and 5-LOX pathway can suppress growth of human lung cell lines more efficiently than inhibition of either pathway alone.

In this study, we show that a combination of clinically relevant concentrations of the COX-2 inhibitor celecoxib with the 5-LOX inhibitors REV5901, and MK886 was more effective in suppression of growth and induction of death than each of the agents alone or in dual combination. Furthermore, dual inhibition of COX-2 and 5-LOX activities by celecoxib and REV5901 produced a significant greater response than treatment with the individual inhibitors alone. We observed approximately 10-fold greater growth-inhibitory effects on a molar basis with the FLAP inhibitor MK886 than what was achieved with the

selective enzyme inhibitors celecoxib or REV5901. MK886 inhibits the translocation of 5-LOX by binding to the active site of FLAP, which is required for the enzymatic activity of 5-LOX[31]. It has also been reported to induce apoptosis independently of both 5-LOX and FLAP[32]. In agreement with previous studies on various type of cancer cell lines, we obtained no direct correlation between the expression status of COX-2, FLAP or 5-LOX and the growth-inhibitory activity of celecoxib, MK886 or REV5901, respectively in premalignant and malignant lung cell lines[17,18,21]. However, the expression levels of COX-2, 5-LOX and FLAP in these cells correlated with the production of their major metabolites PGE_2, 5-HETE, and LTB_4, which increased with the degree of malignancy implicating a role in lung cancer development. Numerous studies have demonstrated that PGE_2 is markedly suppressed by celecoxib at concentrations below 5 μM in cell lines expressing the target enzyme COX-2[18,20,23,24,28]. Likewise, inhibition of 5-LOX activity by MK886 completely blocked the production of 5-HETE in human prostate cancer cells and addition of exogenous 5-HETE protected cells from undergoing apoptosis[14]. On the other hand, COX-2 or 5-LOX inhibitors induce apoptosis in cell lines, which lack the target enzymes, suggesting mechanism(s) unrelated to the suppression of the enzyme activity[18,19,21]. However, our data implicate that suppression of the 5-LOX activity is important for induction of cell death in 1198 and A549 cell lines based on partial protection by exogenous addition of 5-HETE to a triple drug combination of COX-2 and 5-LOX inhibitors compared to cells treated identically but without the 5-LOX metabolite. In A549 cells, which express both enzymes COX-2 and 5-LOX, exogenous PGE_2 was also able to rescue some of the cells from death similarly to the effect of 5-HETE while the combination of PGE_2 and 5-HETE in addition to the triple drug treatment was even more effective in preventing cell death than each of the AA metabolites alone.

These findings indicate that the COX-2 and 5-LOX inhibitors induced cell death at least in part through suppression of the production of 5-HETE, PGE_2, or both. Moreover, we found that 1198 premalignant cells were more sensitive than A549 malignant cells suggesting that inhibitors of the AA metabolism might be more effective in lung cancer prevention than in therapy. In fact, Rioux et al. have reported that a combination of the 5-LOX inhibitor A-79175 with the COX inhibitor acetylsalicylic acid reduced tumor multiplicity by 87%, which was the most effective preventive intervention compared to the inhibition of either pathway alone using a mice model of lung carcinogenesis[16].

In conclusion, combinations of low doses of biochemical inhibitors targeting related AA-metabolizing pathways support a rational approach in lung cancer prevention and therapy that warrant further investigations.

REFERENCES

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. *CA Cancer J Clin* 55:10-30, 2005.
2. Bunn PA Jr, Keith RL. The future of cyclooxygenase-2 inhibitors and other inhibitors of the eicosanoid signal pathway in the prevention and therapy of lung cancer. *Clin Lung Cancer* 3:271-277, 2002.
3. Laskin JJ, Sandler AB. The importance of the eicosanoid pathway in lung cancer. *Lung Cancer* 41 Suppl. 1:S73-79, 2003.
4. Sharma S, Yang SC, Zhu L, Reckamp K, Gardner B, Baratelli F, et al. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer Res* 65(12):5211-5220, 2005.
5. Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 58:3761-3764, 1998.
6. Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, Ristimaki A. Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res* 58:4997-5001, 1998.
7. Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. *Clin Cancer Res* 7:861-867, 2001.
8. Hosomi Y, Yokose T, Hirose Y, Nakajima R, Nagai K, Nishiwaki Y, et al. Increased cyclooxygenase 2 (COX-2) expression occurs frequently in precursor lesions of human adenocarcinoma of the lung. *Lung Cancer* 30:73-81, 2000.

9. Funk CD. Prostaglandins and leukotriens: advances in eicosanoid biology. *Science* 294:1871-1875, 2001.

10. Wang D, Dubois RN. Prostaglandins and cancer. *Gut* 55:115-122, 2006.

11. Tang DG, Chen YQ, Honn KV. Arachidonate lipoxygenase as essential regulators of cell survival and apoptosis. *Proc Natl Acad Sci USA* 93:5241-5246, 1996.

12. Romano M, Claria J. Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. *FASEB J* 17:1986-1995, 2003.

13. Avis IM, Jett M, Boyle T, Vos MD, Moody T, Treston AM, et al. Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. *J Clin Invest* 97:806-813, 1996.

14. Ghosh J, Myers CE. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc Natl Acad Sci USA* 95:13182-13187, 1998.

15. Moody TW, Leyton J, Martinez A, Hong S, Malkinson A, Mulshine JL. Lipoxygenase inhibitors prevent lung carcinogenesis and inhibit non-small cell lung cancer growth. *Exp Lung Res* 24:617-628, 1998.

16. Rioux N, Castonguay A. Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. *Carcinogenesis* 19:1393-1400, 1998.

17. Hong SH, Avis I, Vos MD, Martinez A, Treston AM, Mulshine JL. Relationship of arachidonic acid metabolizing enzyme expression in epithelial cell lines to the growth effects of selective biochemical inhibitors. *Cancer Res* 59:2223-2228, 1999.

18. Schroeder CP, Yang P, Newman RA, Lotan R. Eicosanoid metabolism in squamous cell carcinoma cell lines derived from primary and metastatic head and neck cancer and its modulation by celecoxib. *Cancer Biol Ther* 9:29-34, 2004.

19. Štika J, Vondráček J, Hofmanová J, Šimek V, Kozubík A. MK-886 enhances tumor necrosis factor-α-induced differentiation and apoptosis. *Cancer Lett* 215:129-140, 2004.

20. Gregor JI, Kilian M, Heukamp I, Kiewert C, Kristiansen G, Schimke I, et al. Effects of selective COX-2 and 5-LOX inhibition on prostaglandin and leukotriene synthesis in ductal pancreatic cancer in syrian hamster. *Prostaglandins Leuk Essent Fatty Acids* 73:89-97, 2005.

21. Sun SY, Schroeder CP, Yue P, Lotan D, Hong WK, Lotan R. Enhanced growth inhibition and apoptosis induction in NSCLC cell lines by combination of celecoxib and 4HPR at clinically relevant concentrations. *Cancer Biol Ther* 4:407-413, 2005.

22. Mao JT, Tsu IH, Dubinett SM, Adams B, Sarafian T, Baratelli F, et al. Modulation of pulmonary leukotriene B4 production by cyclooxygenase-2 inhibitors and lipopolysaccharide. *Clin Cancer Res* 10:6872-6878, 2004.

23. Ye YN, Wu WK, Shin VY, Bruce IC, Wong BC, Cho CH. Dual inhibition of 5-LOX and COX-2 suppresses colon cancer formation promoted by cigarette smoke. *Carcinogenesis* 26:827-834, 2005.

24. Li N, Sood S, Wang S, Fang M, Wang P, Sun Z, et al. Overexpression of 5-lipoxygenase and cyclooxygenase 2 in hamster and human oral cancer and chemopreventive effects of zileuton and celecoxib. *Clin Cancer Res* 11:2089-2096, 2005.

25. Klein-Szanto AJ, Iizasa T, Momiki S, Garcia-Palazzo I, Caamano J, Metcalf R, et al. A tobacco-specific N-nitrosamine or cigarette smoke condensate causes neoplastic transformation of xenotransplanted human bronchial epithelial cells. *Proc Natl Acad Sci* USA 89:6693-6697, 1992.

26. Reddel RR, Ke Y, Gerwin BI, McMenamin MG, Lechner JF, Su RT, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res* 48:1904-1909, 1988.

27. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl Cancer Inst* 82: 1107-1112, 1990.

28. Kempen EC, Yang P, Felix E, Madden T, Newman RA. Simultaneous quantification of arachidonic acid metabolites in cultured tumor cells using high performance liquid: chromatography/electrospray ionization tandem mass spectrometry. *Anal Biochem* 297:183-190, 2001.

29. Yang P, Chan D, Felix E, Cartwright C, Menter DG, Madden T, Klein RD, Fischer SM, Newman RA. Formation and antiproliferative effect of prostaglandin E(3) from eicosapentaenoic acid in human lung cancer cells. *J Lipid Res* 45(6):1030-1039, 2004.

30. Lu S, Zhang X, Badawi AF, El-Sohemy A, Archer MC. Cyclooxygenase-2 inhibitor celecoxib inhibits promotion of mammary tumorigenesis in rats fed a high fat diet rich in n-6 polyunsaturated fatty acids. *Cancer Lett* 184:7-12, 2002.

31. Rouzer CA, Ford-Hutchinson AW, Morton HE, Gillard JW. MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J Biol Chem* 265:1436-1442, 1990.

32. Datta K, Biswal SS, Kehrer JP. The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, induces apoptosis independently of FLAP. *Biochem J* 340:371-375, 1999.

LEGENDS

Figure 1. (A) Production of PGE_2, 5-HETE and LTB_4, and (B) expression of 5-LOX, FLAP, and COX-2 in various cell lines representing an in vitro model of human lung carcinogenesis. Cells were treated in standard medium for 72 h prior to analysis by ESI-LC/MS/MS or separation of a total of 50 µg of protein per sample using 10% SDS-polyacrylamid gel electrophoresis as described under materials and methods. For the measurement of eicosanoid metabolites, culture medium of three representative cell lines (BEAS-2B, 1198, and A549) was processed. The immunoblot was probed with antibodies specific for COX-2, 5-LOX, and FLAP. β-actin served as loading control.

Figure 2. Chemical structure of the selective COX-2 inhibitor celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide, the FLAP inhibitor MK886 (1[(4-chlorophenyl)methyl]-3-1,1-dimethyl)thio]-α,α-dimethylethyl)-3-(1-methyl)-1H-indole-2-propanoic acid, sodium salt), and the selective 5-LOX inhibitor REV5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol).

Figure 3. Effects of (A) celecoxib, (B) MK886, or (C) REV5901 on growth suppression of various cell lines representing an in vitro model of human lung carcinogenesis. Cell lines were treated with the inhibitors for 72 h before determination of cell number using the SRB assay as described in materials and methods. Concentrations required for the determination of IC_{50} values (right panels) were obtained by interpolation of concentration response curves. Results represent mean ± SD of three independent experiments with no drug added serving as control and expressed as 100% cell growth.

Figure 4. Effects of celecoxib (5 μM), MK886 (1 μM), REV5901 (5 μM), and their combinations on growth of 1198 premalignant (full bars) and A549 malignant (open bars) lung cell lines. The cells were seeded in 96-well culture plates and allowed to adhere overnight before treatment with the inhibitors. Changes in cell growth was determined by the SRB assay after 72 h of incubation. Results are expressed as mean ± SD (n=3) with no inhibitor added serving as control and expressed as 100% growth. *$P<0.05$, vs untreated cells determined using Student's paired *t*-test.

Figure 5. Induction of cell death by celecoxib (5 μM), MK886 (1 μM), REV5901 (5, and 7.5 μM, respectively) alone or in combination and the effects of PGE_2 (0.5 μg/ml), and/or 5-HETE (100 nM) in (A) 1198 and (B) A549 cell lines. The cells were treated with the inhibitors, and/or PGE_2, and 5-HETE for 72 h before collection of detached and adherent cells and studied for cell surface Annexin V binding to phosphatidylserin by flow cytometry. Control cultures were treated with medium containing solvent vehicle only (0.02% v/v DMSO). Data shown are representative of three independent experiments.

Figure 1.

Schroeder CP et al.

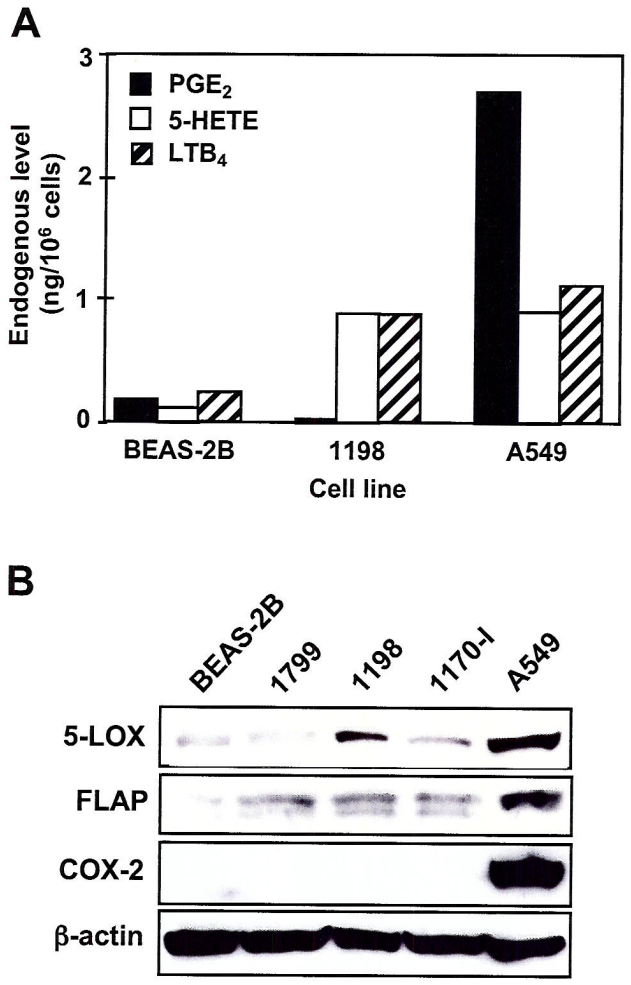
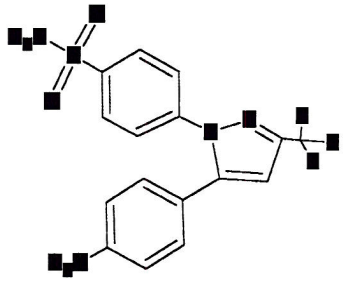
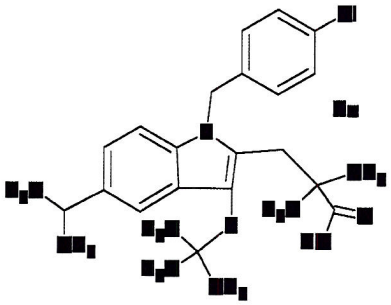


Figure 2.

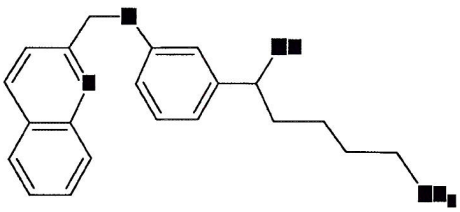
Schroeder CP et al.



Celecoxib
4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide



MK886
1-[(4-chlorophenzyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl-1H-indole-2-propanoic acid, sodium salt



REV5901
α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol

Figure 3.

Schroeder CP et al.

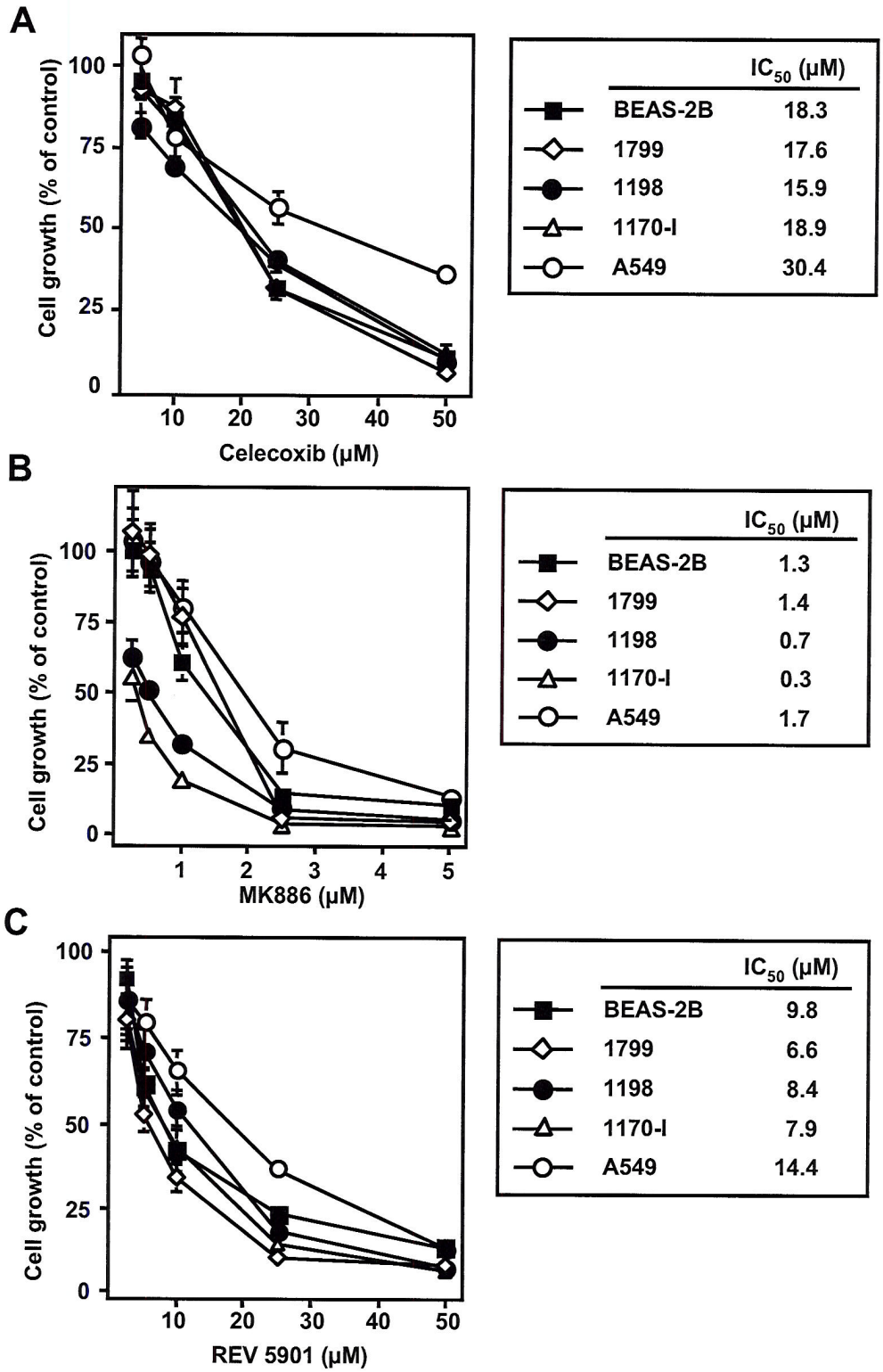


Figure 4.

Schroeder CP et al.

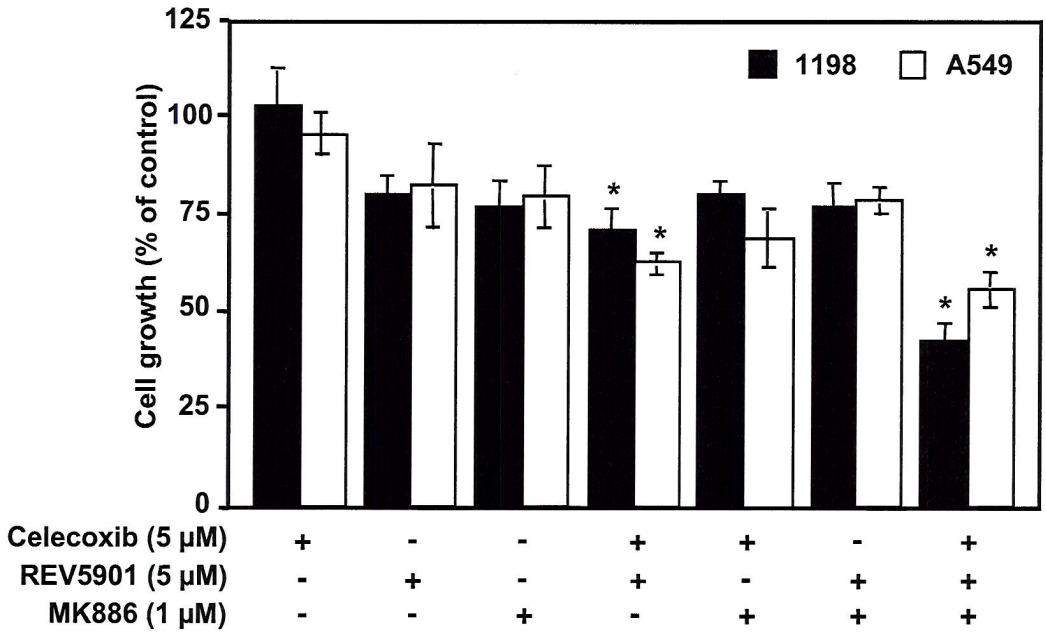
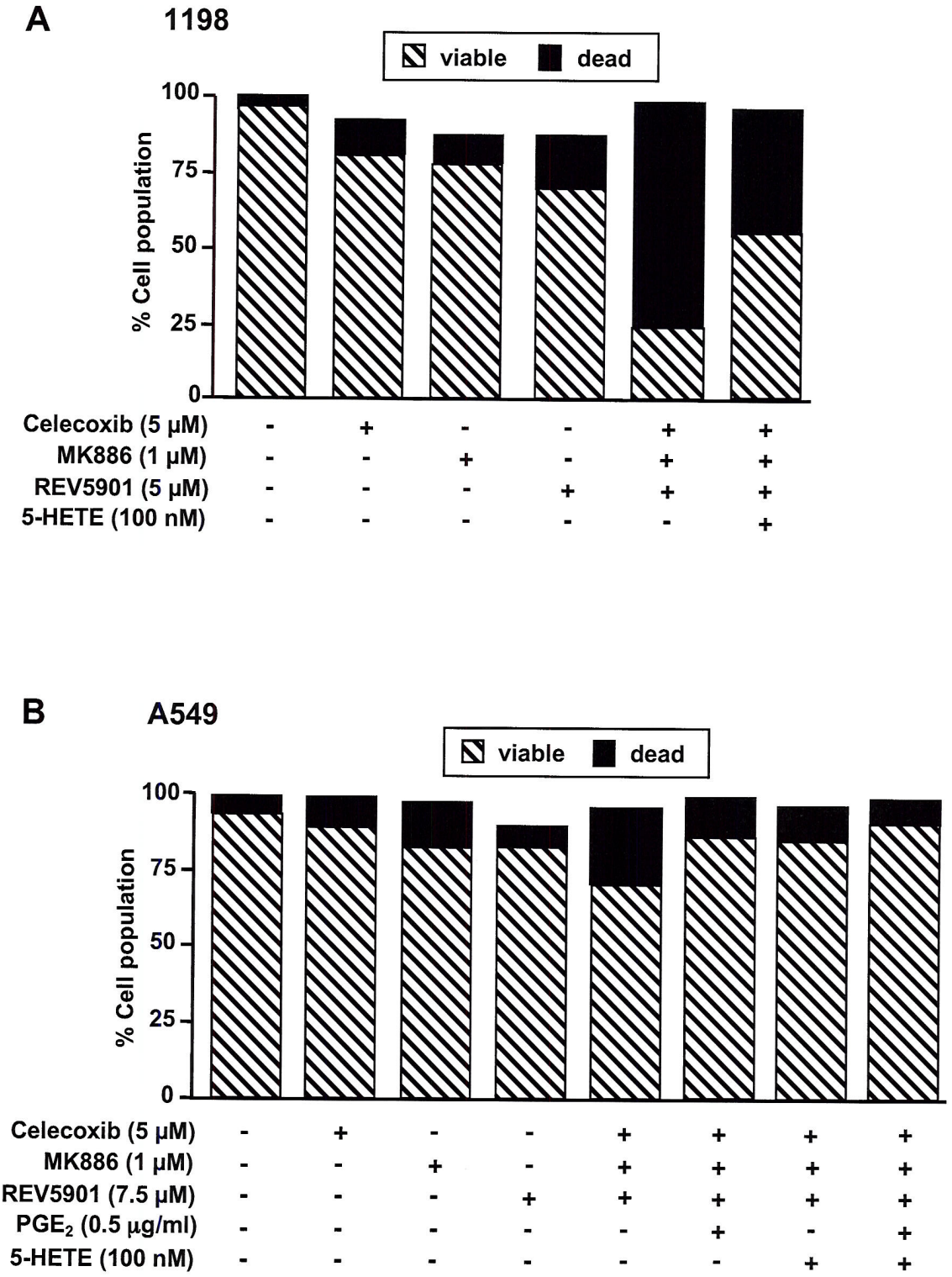


Figure 5.

Schroeder CP et al.



Down-regulation of Hepatoma-Derived Growth Factor Inhibits Anchorage-Independent Growth and Invasion of Non–Small Cell Lung Cancer Cells

Jun Zhang,[1,4] Hening Ren,[1] Ping Yuan,[1] Wenhua Lang,[1] Li Zhang,[2] and Li Mao[1,3]

Departments of [1]Thoracic/Head and Neck Medical Oncology and [2]Biostatisitcs, The University of Texas M.D. Anderson Cancer Center; [3]Cancer Biology Program, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas; and [4]Department of Thoracic Surgery, First Clinical College, China Medical University, Shenyang, PR China

Abstract

We recently reported that a high level of hepatoma-derived growth factor (HDGF) expression in tumors correlates with a high incidence of tumor relapse or distant metastasis and shortened survival time in patients with non–small cell lung cancer (NSCLC). However, the mechanisms of the HDGF-associated aggressive biological behavior are unknown. In this study, we knocked down HDGF expression in NSCLC cells to determine the biological consequences. Transfection with *HDGF*-specific small interfering RNA (siRNA) resulted in down-regulation of HDGF expression in four NSCLC cell lines. Down-regulation of HDGF resulted in no detectable effect on anchorage-dependent cell growth as determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, a microelectronic cell sensor system, and flow cytometry. In contrast, cells transfected with HDGF-siRNA grew more slowly and formed significantly fewer colonies in soft agar than did cells treated with LipofectAMINE alone or transfected with negative control siRNA. In an *in vitro* invasion assay, significantly fewer cells transfected with HDGF-siRNA than cells treated with LipofectAMINE alone were able to invade across a Matrigel membrane barrier. In an *in vivo* mouse model, A549 cells treated with HDGF-siRNA grown significantly slower than the cells treated with LipofectAMINE alone or negative control siRNA. Morphologically, HDGF-siRNA–treated tumors exhibited markedly reduced blood vessel formation and increased necrosis, whereas the Ki67 labeling indices were similar in tumors treated with controls. Our results suggest that HDGF is involved in anchorage-independent growth, cell invasion, and formation of neovasculature of NSCLC. These qualities may contribute to the HDGF-associated aggressive biological behavior of NSCLC. (Cancer Res 2006; 66(1): 18-23)

Introduction

Hepatoma-derived growth factor (HDGF) is a heparin-binding growth factor originally purified from media conditioned with the human hepatoma cell line HuH-7 and can stimulate proliferation of Swiss 3T3 cells (1). Its precise function is unclear, but HDGF is known to be highly expressed during the early development of many tissues, including cardiovascular (2), kidney (3), and liver (4). Although lacking the secretory sequence present in most secretory proteins (5), HDGF has been shown to act as a potent exogenous mitogen for HuH-7 hepatoma cells (6), COS-7 kidney cells (6), aortic vascular smooth muscle cells (7), and endothelial cells (3). As deduced from the cDNA clone of *HDGF*, the amino acid sequence contains 240 residues with a motif homologous to the consensus sequences of a bipartite nuclear localization sequence and a DNA-binding PWWP motif, suggesting that the protein translocates to the nucleus and binds to DNA. In fact, HDGF is found mainly in nucleus, and its role as a transcription factor has been postulated (8, 9).

In a recent study, we investigated the role of HDGF in non–small cell lung cancer (NSCLC) and found that the protein is frequently overexpressed in these tumors (10). In patients with early-stage NSCLC, poorer clinical outcome was significantly correlated with higher HDGF expression, suggesting that HDGF is involved in the determination of aggressive biological behavior of NSCLC cells. To elucidate the mechanism of HDGF-mediated aggressiveness in NSCLC cells, we down-regulated *HDGF* expression in these cells using small interfering RNA (siRNA) technology and studied effects of the down-regulation in cell proliferation and invasion. Our results suggest that HDGF is involved in anchorage-independent growth and cell invasion of NSCLC. These qualities may contribute to the HDGF-associated aggressive biological behavior of NSCLC.

Materials and Methods

Cell culture. NSCLC cell lines H226, H1944, H292, H157, A549, H596, H460, and H358 were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). The normal human bronchial epithelial cell lines HBE1 and HBE3 (kindly provided by Dr. John Minna of The University of Texas Southwestern Medical Center, Dallas, TX) were cultured in keratinocyte serum-free medium with 25 μg/mL bovine pituitary extract and 0.2 ng/mL recombinant epidermal growth factor (Invitrogen, Carlsbad, CA).

siRNA and knockout of *HDGF* expression. We selected two sites in the *HDGF* mRNA sequence as siRNA targets based on principles described previously (11). The targeted *HDGF* sequences, based on which the siRNAs were chemically synthesized by Ambion (Austin, TX), were 5′-AACCGGCA-GAAGGAGUACAAA-3′ (siRNA-1) and 5′-AAAUCAACAGCCAACAAAUAC-3′ (siRNA-2). The negative control siRNAs were purchased from Ambion. *In vitro* transfections were done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following manufacturer's protocols.

Cell proliferation analysis. Cells were plated onto 96-well plates at a density of 1×10^4 per well with medium containing 10% FBS and incubated for 15 hours. Cell numbers were determined at 0, 24, 48, and 72 hours after transfection using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–based CellTiter96 cell proliferation assay (Promega, Madison, WI). ACEA RT-CES (ACEA Biosciences, San Diego, CA), a microelectronic cell sensor system, was used to confirm the number of living cells. NSCLC cells (1×10^4) were seeded into each sensor-containing well

Requests for reprints: Li Mao, Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Unit 432, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-6363; Fax: 713-796-8655; E-mail: lmao@mdanderson.org.

doi:10.1158/0008-5472.CAN-04-3905

(19.6-mm^2 surface with 150 μL of medium) of the microtiter plates. The electronic sensors provided a continuous (every 6 hours), quantitative measurement of the cell index (reflect to the surface area covered by the cells) in each well. After 15 hours of culture, the cells were transfected as described above. Cell growth was measured every 6 hours for 72 hours, and cell indexes were recorded for each well at all time points.

Cell cycle analysis. A549 cells were harvested by trypsinization 72 hours after transfection and fixed with 70% ethanol. After RNase treatment, the cell cycle distribution was determined using a BD FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA).

Anchorage-independent growth assay. Twenty-four hours after transfection with siRNA, ~2,000 cells in 1 mL of 0.3% agarose with DMEM were plated in each well on the top of existing 0.6% bottom agarose in six-well tissue culture plates in triplicate for each treatment condition. The plates were covered with 1 mL of medium with 10% FBS and incubated at 37°C in a 5% CO_2 incubator for 3 weeks. The covering medium was replaced every week. At the end of 3 weeks, cell colonies >0.1 mm in diameter were counted under a microscopic field at ×40 magnifications. Means were based on numbers from triplicate wells for each treatment condition and were analyzed using two-sided Student's *t* test.

***In vitro* cell invasion assay.** The *in vitro* invasion assay was carried out in BD BioCoat Matrigel invasion chambers (Becton Dickinson). After rehydration of the chambers, 1.1×10^4 cells in 100 μL of the growth medium with 10% FBS were added into each of the upper chambers. Cells in the chambers were transfected with LipofectAMINE alone or 100 nmol/L HDGF-siRNA-1 in serum-free condition. Four hours later, the medium was replaced with fresh growth medium containing 10% FBS in the upper chambers, whereas the lower wells contained serum-free medium. After 20 hours, the medium in each of the lower wells was replaced with 750 μL of serum-free medium containing 30 μg/mL laminin (Sigma-Aldrich, St. Louis, MO). After an additional 24 hours of incubation, the noninvading cells on the upper side of the chamber membranes were removed. The invading cells to the opposite side of the chamber membranes were examined. The invading cells on each of triplicate membranes were counted. Means were based on the numbers from the triplicate wells for each treatment condition and were analyzed using two-sided Student's *t* test.

Western blot analysis. Total proteins were loaded into each well on 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). The membranes were probed with a rabbit polyclonal anti-HDGF antibody (gift from Dr. Allen Everett of Johns Hopkins Hospital, Baltimore, MD) followed by incubating with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Amersham Biosciences). Immunodetection was done using the enhanced chemiluminescence Western blotting analysis system (Amersham Biosciences). β-Actin was used as protein loading control monoclonal anti-β-actin antibody (Sigma-Aldrich).

Global gene expression analysis. Total RNA was isolated from cells using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Ten micrograms of total RNA were reverse-transcribed into double-stranded cDNA and then transcribed in the presence of biotin-labeled ribonucleotides, using the BioArray High-Yield RNA transcript labeling kit (Enzo Laboratories, Farmingdale, NY) as described by the manufacturer. The biotin-labeled cRNA was purified using RNeasy mini-column (RNeasy kit, Qiagen) and fragmented at 94°C for 35 minutes in 1× fragmentation buffer [40 mmol/L Tris-acetate (pH 8.0), 100 mmol/L KOAc, 30 mmol/L MgOAc]. Affymetrix U133A chips were used for gene expression analysis using the Affymetrix GeneChip system (Affymetrix, Santa Clara, CA).

The expression levels were extracted from positional-dependent nearest-neighbor model developed by Zhang et al. (12). Genes that are absent or always expressed at low levels were excluded from further analysis because variation in gene expression at low levels are usually not reproducible. The removed genes have mean log expression level of <7.1. Genes with SD <0.06 were also removed. Comparisons were then done between cells treated with LipofectAMINE alone and cells treated with 100 nmol/L HDGF-siRNA-1.

Northern blot analysis. Total RNA (10 μg) was loaded in each lane. cDNA probes corresponding to *HDGF, GLO1, SERPINE2, AXL*, and actin were prepared using reverse transcription-PCR followed by cDNA purification and labeling.

***In vivo* tumor model.** Athymic Swiss *nu/nu*/Ncr nude (*nu/nu*) mice, bred and maintained in our institutional specific pathogen-free mouse colony, were used. Briefly, 4-week-old male nude mice were injected s.c. with 10^6 A549 cells in 100 μL of PBS at a single dorsal site. Three groups (five each) of mice were tested. Group 1 were injected with A549 cells treated with LipofectAMINE alone; group 2 were injected with A549 cells treated with LipofectAMINE plus 100 nmol/L HDGF-siRNA-1; group 3 was injected with A549 cells treated with LipofectAMINE plus 100 nmol/L negative control siRNA. Tumor size was measured every 2 days for 20 days. Tumor

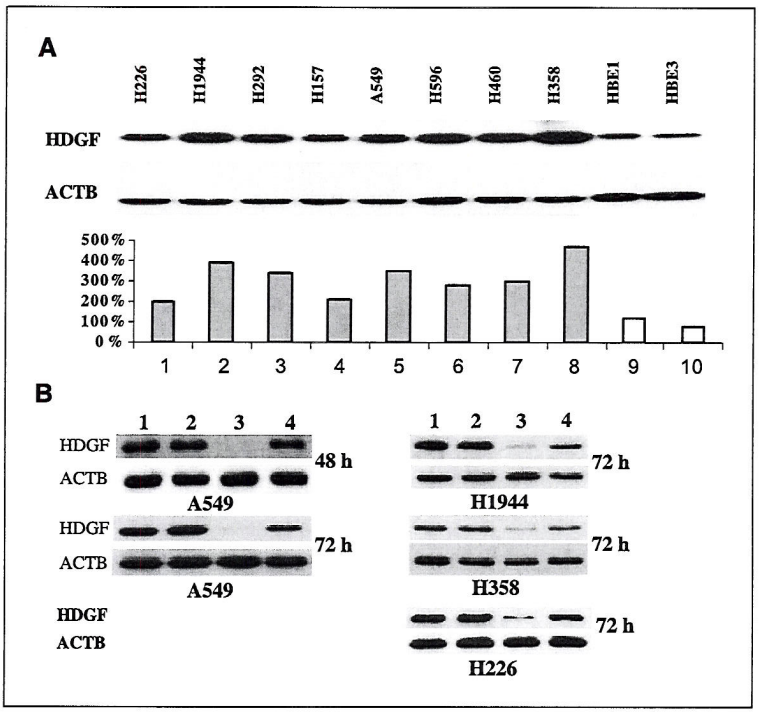


Figure 1. *A,* Western blots showing expression of HDGF protein in eight NSCLC cell lines and two immortalized normal bronchial epithelial cell lines. β-Actin (*ACTB*) served as protein loading control. *Bottom,* relative expression level of HDGF quantified based its β-actin level. *B,* down-regulation of HDGF protein expression induced by HDGF-siRNA-1 in A549 cells (48 and 72 hours after siRNA administration) and in H1944, H358, and H226 cells (72 hours after siRNA administration). β-Actin served as protein loading control. *Lane 1,* treated with LipofectAMINE alone; *lane 2,* treated with 100 nmol/L negative control siRNA; *lane 3,* treated with 100 nmol/L HDGF-siRNA-1; *lane 4,* treated with 100 nmol/L HDGF-siRNA-2.

growth was quantified by measuring the tumors in three dimensions with calipers. The results were expressed as the mean tumor volume (n = 5) with 95% confidence intervals. The statistical significance of differences in tumor growth was analyzed using Wilcoxon rank sum test.

Tumor morphology and Ki67 immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections were stained with H&E for morphologic examination. For Ki67 immunohistochemistry, an anti-Ki67 antibody (Lab Vision, Fremont, CA) was used. The expression signal was detected using standard avidin-biotin immunohistochemical techniques according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA).

Results and Discussion

HDGF is highly expressed in NSCLC. Western blot analysis using a polyclonal anti-HDGF antibody revealed that most of the NSCLC cell lines expressed high levels of HDGF, whereas the immortalized normal bronchial epithelial cell lines (HBE1 and HBE3) expressed low levels of HDGF (Fig. 1*A*). We selected four cell lines (A549, H358, H226, and H1944) for further investigation.

HDGF-siRNA-1 knocks out *HDGF* in NSCLC cells. To determine the role of HDGF in NSCLC, we used RNA interference (RNAi) strategy to down-regulate the molecule. In A549 cells, the HDGF protein level was substantially reduced 48 hours after transfection with 100 nmol/L HDGF-siRNA-1, whereas 100 nmol/L HDGF-siRNA-2 induced only a slight reduction of the protein; these effects lasted up to at least 72 hours after transfection (Fig. 1*B*). Using 100 nmol/L concentration of HDGF-siRNA-1, the protein level was similarly down-regulated in H1944, H358, and H226 cells. These results indicate that HDGF-siRNA-1 effectively and specifically down-regulated HDGF protein expression in a panel of NSCLC cells.

Down-regulation of *HDGF* has minimal effect on anchorage-dependent growth of NSCLC cells. We next examined the growth curves of A549 cells transfected with 2 or 100 nmol/L HDGF-siRNA-1 in the presence of 5% bovine serum. Results of the MTT assay showed that the growth curves of these cells were comparable to those of cells treated with LipofectAMINE alone or transfected with negative control siRNA (Fig. 2*A*). These observations were confirmed by using a microelectronic cell sensor system (Fig. 2*B*). Similar results were obtained in H1944, H358, and H226 cells (data not shown). These data suggest that HDGF plays a minimal role in controlling anchorage-dependent growth in NSCLC cells in our culture condition.

To determine a role of HDGF in cell cycle regulation, we did flow cytometry analysis in A549 cells 72 hours after transfection with 2 or 100 nmol/L HDGF-siRNA-1. The cell cycle distributions of these cells were similar to those of cells treated with LipofectAMINE alone or transfected with negative control siRNA (Fig. 2*C*).

Although HDGF can stimulate DNA synthesis and cell proliferation in vascular or bronchial epithelial cells has been previously reported (8, 9, 13), our results are consistent with our clinical observation that the expression levels of HDGF was not associated with Ki67 labeling indices in primary NSCLC (10). In fact, HDGF-mediated cell growth was observed only when the cells were cultured in serum-free condition (13, 14); the presence of serum would have masked HDGF stimulation because of the effect of other growth stimulators in serum.

Down-regulation of *HDGF* reduces anchorage-independent growth of NSCLC cells. The effect of HDGF on anchorage-independent growth of the four cell lines was analyzed using the soft agar growth assay. Three weeks after seeding, cells transfected with 100 nmol/L HDGF-siRNA-1 produced significantly fewer and smaller colonies than did cells treated with LipofectAMINE alone or transfected with negative control siRNA (Fig. 3*A*). The numbers (average of triplicate wells with three randomly selected fields per well) of colonies visible in a microscopic field at ×40 magnifications for the four cell lines are presented in an attached table (Fig. 3*A*). These results suggest that HDGF is involved in anchorage-independent cell growth, a feature of malignant transformation, of NSCLC cells.

Down-regulation of *HDGF* reduces NSCLC cells' capability to invade. We then used Matrigel invasion chambers to determine the effect of HDGF on the invasion potential of the four cell lines. Transfection with 100 nmol/L HDGF-siRNA-1 resulted in significantly fewer invasive cells in A549 and H226 cell lines (Fig. 3*B*). The number of cells transfected with HDGF-siRNA-1 that were invasive averaged 140 (140 ± 73.65), whereas the number of cells treated with LipofectAMINE alone that were invasive averaged 759 (759 ± 156.79; P = 0.004) for A549; 126 (126 ± 28) versus 516 (516 ± 19; P = 0.0001) for H226. Because H1944 and H358 did not invade in both controls and treated cells in these chambers, we were unable to determine the effect of HDGF-siRNA-1 in these cell lines.

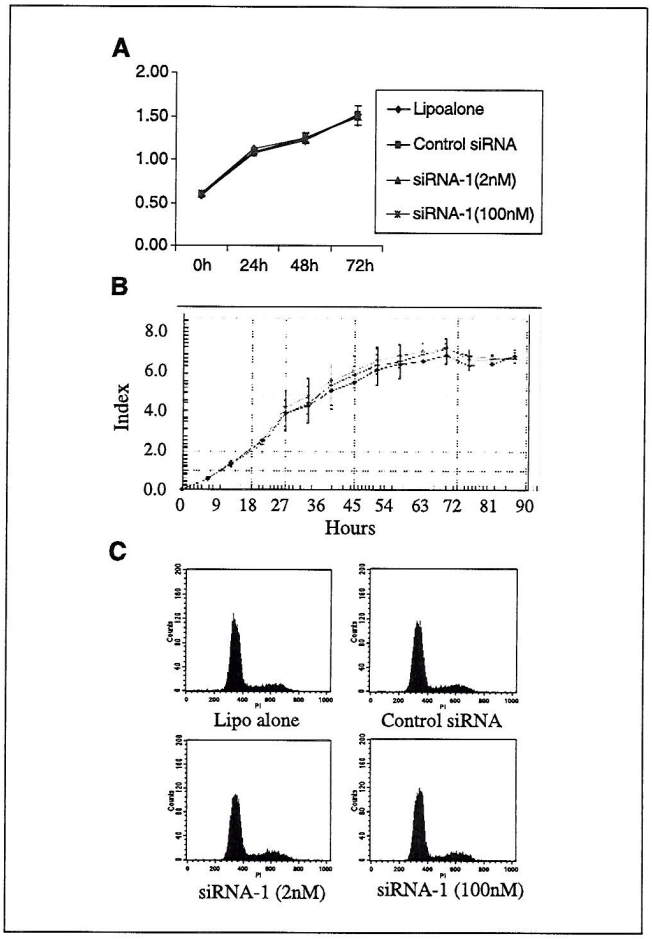
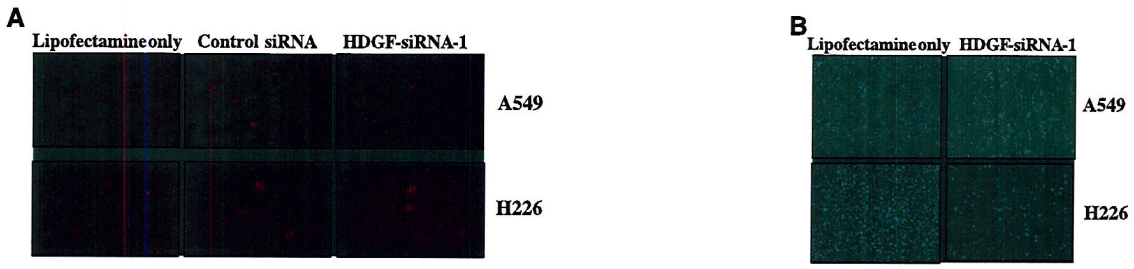


Figure 2. Effect of *HDGF* down-regulation on anchorage-dependent cell proliferation of A549 cells, as measured by MTT assay (*A*); a microelectronic cell sensor system (*B*), where the red line represents cells treated with LipofectAMINE alone, the green line for cells treated with 2 nmol/L HDGF-siRNA-1, and the blue line for cells treated with 100 nmol/L HDGF-siRNA-1; and flow cytometry 72 hours after siRNA administration (*C*). *Points,* means; *bars,* SD (*A* and *B*).

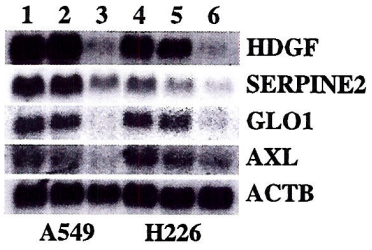


Cell Line	Lipofectomine	Control siRNA	HDGF-siRNA-1	P value (t-test) (Control/HDGF)
A549	12.11±2.22	13.44±0.51	2.89±0.51	0.0001
H1944	9.00 ±1.00	9.67 ±1.53	4.00 ±1.00	0.006
H358	8.00 ±1.00	7.33 ±1.15	2.67 ±0.58	0.003
H226	13.67 ±3.06	14.00 ±2.65	3.00 ±1.00	0.003

Figure 3. *A,* effect of *HDGF* down-regulation on anchorage-independent cell growth of A549 and H226 cells, as measured by soft agar assay (×40 magnifications). *Bottom,* counts of colonies for each of the four cell lines and *P*s of statistical analysis. *B,* invasion capability of A549 cells and H226 cells measured by an *in vitro* cell invasion system (×100 magnifications).

Together with the soft agar experiments, these results may explain the increased rates of tumor relapse and distant metastasis in patients whose primary NSCLC tumors had a high level of HDGF after surgical removal of the tumors (10). An association between higher HDGF and poor clinical outcome has also been observed in patients with NSCLC by another group (15) and in patients with primary hepatocellular carcinoma (16) and melanoma (17).

Genes down-regulated by HDGF-siRNA-1 treatment. We did global gene expression analysis using Affymetrix U133A gene chip, which can measure expression of >16,000 unique genes, to elucidate potential mechanism of HDGF-siRNA-1–induced growth inhibition in soft agar and inhibition of invasion in the invasion chambers. We compared gene expression profiles between A549 cells treated with LipofectAMINE alone and A549 cells treated with 100 nmol/L HDGF-siRNA-1 at 48-hour time point. Among 10,938 unique genes with expression level qualifying our analysis as specified in Materials and Method, 15 genes were down-regulated ≥2-fold in HDGF-siRNA-1–treated cells compared with cells treated with LipofectAMINE alone (attached table in Fig. 4), whereas none of the genes were up-regulated ≥2-fold. Among the



Gene symbol	Chromosomal location
HDGF	1q21-q23
GLO1	6p21
SERPINE2	2q33
E46L	22q13
GNG5	1q22
PLOD2	3q23
LOXL2	8p21
PYGB	20p11
GNG10	9q32
PPP1CC	12q13
AXL	19q13
C14orf78	14q32
GALNT1	18q12
F2RL1	5q13
C1orf24	1q25

Figure 4. Expression of *HDGF, SERPINE2, GLO1,* and *AXL* before and after treatment with LipofectAMINE alone (*lanes 1* and *4*), 100 nmol/L control-siRNA (*lanes 2* and *5*), and 100 nmol/L HDGF-siRNA-1 (*lanes 3* and *6*) in A549 and H226 cells measured by Northern blot analysis. *Right,* top 15 genes down-regulated after HDGF-siRNA-1 treatment measured by Affymetrix U133A chip.

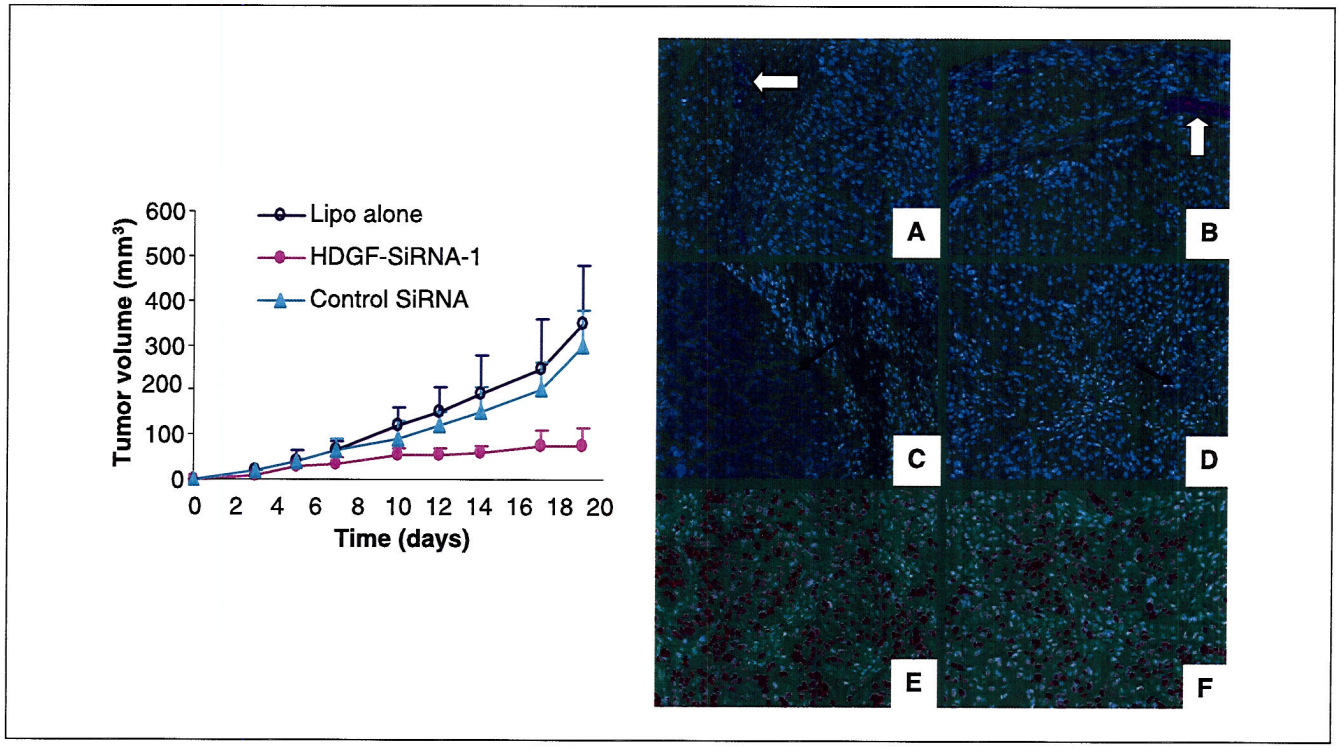


Figure 5. *Left,* effects of *HDGF* down-regulation on A549 NSCLC xenograft tumor growth. The tumor growth curves represent cells treated with LipofectAMINE alone, LipofectAMINE plus 100 nmol/L negative control siRNA, and LipofectAMINE plus 100 nmol/L HDGF-siRNA-1, respectively, as labeled. *Point,* mean tumor volume (calculated from five mice); *bars,* upper 95% confidence intervals. *Right, A-D,* H&E-stained tumor sections. *E* and *F,* tumor sections stained with Ki67 immunohistochemically. *A,* tumor treated with LipofectAMINE alone; *B,* tumor treated with 100 nmol/L negative control siRNA; *C* and *D,* tumors treated with 100 nmol/L HDGF-siRNA-1; *E,* tumor treated with LipofectAMINE alone; *F,* tumor treated with 100 nmol/L HDGF-siRNA-1. *Open arrows,* blood vessels (*A* and *B*); *black arrows,* areas of tumor necrosis (*C* and *D*).

15 genes, the expression of *HDGF* was down-regulated most dramatically (>4-fold) as expected. The next two genes are *GLO1* and *SERPINE2*. GLO1 has been shown elevated in lung cancers (18), whereas SERPINE2 has been suggested to play a role in invasion of pancreatic cancer cells (19). AXL, a receptor tyrosine kinase also in the list, has been reported overexpressed in multiple types of cancers (20–22) and linked to adverse clinical outcome in patients with cancer (23). To confirm the down-regulation of *GLO1, SERPINE2*, and *AXL* in HDGF-siRNA-1 treated cells, we did Northern blot analysis to compare the gene expression levels in A549 and H226 cells treated with HDGF-siRNA-1, siRNA control, and LipofectAMINE alone. The results are consistent with the microarray experiment (Fig. 4) and agree with the notion that *HDGF* is involved in regulation of expression of these genes.

Down-regulation of *HDGF* inhibits tumorigenicity of NSCLC cells *in vivo*. To further determine a role of HDGF in progression of NSCLC, we did an *in vivo* animal experiment. We found that A549 cells transfected with 100 nmol/L HDGF-siRNA-1 formed substantially smaller tumors in nude mice compared with those transfected with LipofectAMINE alone or negative control siRNA (Fig. 5, *left*). The tumor volume for mice with cells transfected with the HDGF-siRNA was 76.27 $\pm$ 39.06 mm^3 compared with 345.64 $\pm$ 135.67 or 295.33 $\pm$ 80.53 mm^3 for mice with cells treated with LipofectAMINE alone or negative control siRNA, respectively ($P = 0.037$ or $P = 0.018$, respectively). Under light microscopy, we observed a substantially reduced blood vessels in the HDGF-siRNA–transfected tumors compared with the tumors derived from cells treated with Lipo-fectAMINE or negative control siRNA (Fig. 5*A-D, right*). Substantial tumor necrosis was observed only in tumors derived from cells treated with the HDGF-siRNA (Fig. 5*C* and *D, right*). Interestingly, Ki67 expression index, an indicator of cell proliferation, was similar in tumors of the three animal groups (Fig. 5*E* and *F, right*).

The *in vivo* animal experiment provides a strong support for the importance of HDGF in NSCLC and suggests that HDGF may be a target for treating NSCLC or preventing the development of lung cancer. The finding of reduced blood vessel formation in the HDGF-siRNA–treated tumors suggests that HDGF plays a role in the neovasculature formation *in vivo*, which may be an important mechanism of HDGF in tumor development and progression of NSCLC. This is consistent with previous reports supporting the role of HDGF in angiogenesis as a potent endothelial mitogen and regulator of endothelial cell migration by mechanisms distinct from those used by vascular endothelial growth factor (14, 24). The observed tumor necrosis is likely a consequence of the poor blood supply in these tumors. Consistent with our *in intro* and clinical observations, the lack of change in cell proliferation in the tumors indicates that HDGF plays a minimal role in the tumor cell proliferation for patients with NSCLC. Future studies will focus on the molecular mechanisms of HDGF-induced tumor development and progression as well as on strategies to down-regulate the protein or inhibit its function for potential therapeutic applications.

Acknowledgments

Received 10/29/2004; revised 10/24/2005; accepted 11/11/2005.

Grant support: Department of Defense grant DAMD17-01-1-01689-1 and National Cancer Institute grants PO1 CA106451, PO1 CA91844, and U01 CA86390.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Nakamura H, Kambe H, Egawa T, et al. Partial purification and characterization of human hepatoma-derived growth factor. Clin Chim Acta 1989;183:273–84.
2. Everett AD. Identification, cloning, and developmental expression of hepatoma-derived growth factor in the developing rat heart. Dev Dyn 2001;222:450–8.
3. Oliver JA, Al-Awqati Q. An endothelial growth factor involved in rat renal development. J Clin Invest 1998;102: 1208–19.
4. Enomoto H, Yoshida K, Kishima Y, et al. Hepatoma-derived growth factor is highly ex-pressed in developing liver and promotes fetal hepatocyte proliferation. Hepatology 2002;36:1519–27.
5. von Heijne G. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14: 4683–90.
6. Nakamura H, Izumoto Y, Kambe H, et al. Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor. Its homology with high mobility group-1 protein. J Biol Chem 1994;69: 25143–9.
7. Everett AD, Lobe DR, Matsumura ME, Nakamura H, McNamara CA. Hepatoma-derived growth factor stimulates smooth muscle cell growth and is expressed in vascular development. J Clin Invest 2000;105:567–75.
8. Everett AD, Stoops T, McNamara CA. Nuclear targeting is required for hepatoma-derived growth factor-stimulated mitogenesis in vascular smooth muscle cells. J Biol Chem 2001;276:37564–8.
9. Kishima Y, Yamamoto H, Izumoto Y, et al. Hepatoma-derived growth factor stimulates cell growth after translocation to the nucleus by nuclear localization signals. J Biol Chem 2002;277:10315–22.
10. Ren H, Tang X, Lee JJ, et al. Expression of hepatoma-derived growth factor is a strong prognostic predictor for patients with early-stage non-small-cell lung cancer. J Clin Oncol 2004;22:3230–7.
11. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 2001;20:6877–88.
12. Zhang L, Miles MF, Aldape KD. A model of molecular interactions on short oligonucleotide microarrays. Nat Biotechnol 2003;21:818–21.
13. Mori M, Morishita H, Nakamura H, et al. Hepatoma-derived growth factor is involved in lung remodeling by stimulating epithelial growth. Am J Respir Cell Mol Biol 2004;30:459–69.
14. Everett AD, Narron JV, Stoops T, Nakamura H, Tucker A. Hepatoma-derived growth factor is a pulmonary endothelial cell-expressed angiogenic factor. Am J Physiol Lung Cell Mol Physiol 2004;286: L1194–201.
15. Iwasaki T, Nakagawa K, Nakamura H, Takada Y, Matsui K, Kawahara K. Hepatoma-derived growth factor as a prognostic marker in completely resected non-small-cell lung cancer. Oncol Rep 2005;13:1075–80.
16. Hu TH, Huang CC, Liu LF, et al. Expression of hepatoma-derived growth factor in hepatocellular carcinoma. Cancer 2003;98:1444–56.
17. Bernard K, Litman E, Fitzpatrick JL, et al. Functional proteomic analysis of melanoma progression. Cancer Res 2003;63:6716–25.
18. Sakamoto H, Mashima T, Sato S, Hashimoto Y, Yamori T, Tsuruo T. Selective activation of apoptosis program by *S-p*-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells. Clin Cancer Res 2001;7:2513–8.
19. Buchholz M, Biebl A, Neebetae A, et al. SERPINE2 (protease nexin I) promotes extracellular matrix production and local invasion of pancreatic tumors *in vivo*. Cancer Res 2003;63:4945–51.
20. Wu CW, Li AF, Chi CW, et al. Clinical significance of AXL kinase family in gastric cancer. Anticancer Res 2002;22:1071–8.
21. Meric F, Lee WP, Sahin A, Zhang H, Kung HJ, Hung MC. Expression profile of tyrosine kinases in breast cancer. Clin Cancer Res 2002;8:361–7.
22. Wimmel A, Glitz D, Kraus A, Roeder J, Schuermann M. Axl receptor tyrosine kinase expression in human lung cancer cell lines correlates with cellular adhesion. Eur J Cancer 2001;37:2264–74.
23. Nakano T, Tani M, Ishibashi Y, et al. Biological properties and gene expression associated with metastatic potential of human osteosarcoma. Clin Exp Metastasis 2003;20:665–74.
24. Okuda Y, Nakamura H, Yoshida K, et al. Hepatoma-derived growth factor induces tumorigenesis *in vivo* through both direct angiogenic activity and induction of vascular endothelial growth factor. Cancer Sci 2003;94: 1034–41.

Expression of ΔDNMT3B Variants and Its Association with Promoter Methylation of *p16* and *RASSF1A* in Primary Non–Small Cell Lung Cancer

Jie Wang,[1,4] Garret Walsh,[2] Diane D. Liu,[3] J. Jack Lee,[3] and Li Mao[1]

[1]Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, [2]Department of Thoracic and Cardiovascular Surgery, and [3]Department of Biostatistics, University of Texas M.D. Anderson Cancer Center, Houston, Texas and [4]Department of Oncology, Beijing Cancer Hospital, Beijing University School of Oncology, Beijing, China

Abstract

Despite the role of DNMT3B in *de novo* DNA methylation, a correlation between DNMT3B expression and promoter DNA methylation has not being established in tumors. We recently reported *ΔDNMT3B*, a subfamily of *DNMT3B*, with seven variants, as the predominant expression forms in non–small cell lung cancer (NSCLC). We hypothesized that expression of the *ΔDNMT3B* variants plays a role in promoter methylation formation during lung tumorigenesis. Expression of seven *ΔDNMT3B* variants was measured in 119 NSCLCs and the corresponding normal lungs using reverse transcription-PCR. The expression patterns of *ΔDNMT3B* variants were analyzed with the status of *p16* and *RASSF1A* promoter methylation in the tumors as well as in patients' clinical variables, including outcomes. Expression of *ΔDNMT3B* variants was detected in 94 of 119 (80%) tumors but in only 22 (18%) of the corresponding normal lungs ($P < 0.0001$). *ΔDNMT3B1*, *ΔDNMT3B2*, and *ΔDNMT3B4* were the most frequently detected transcripts in the tumors (62%, 76%, and 46%, respectively). The expression of *ΔDNMT3B* variants was associated with *p16* and *RASSF1A* promoter methylation in the tumors, but the strongest association was between *ΔDNMT3B4* and *RASSF1A*. Forty-two of 46 (91%) tumors with *RASSF1A* promoter methylation expressed *ΔDNMT3B4* compared with only 13 of 73 (18%) tumors without the promoter methylation ($P < 0.0001$). Strong associations were also observed between expression of the variants in the tumors and in patients' clinical outcomes. Expression of *ΔDNMT3B* variants is common in NSCLC and may play an important role in the development of promoter methylation. (Cancer Res 2006; 66(17): 8361-6)

Introduction

DNA methylation plays an important role in regulation of gene expression, genomic imprinting, and X-chromosome inactivation (1–5). During tumorigenesis, promoter hypermethylation is a major mechanism to inactivate tumor suppressor genes with CpG-rich promoters (6). In non–small cell lung cancer (NSCLC), promoter methylation has been frequently detected in several tumor suppressor genes, such as *p16* and *RASSF1A* (7–10). Although some tumors tend to have more promoters methylated than others, each tumor has distinct profiles of methylated promoters, suggesting a complex mechanism in controlling the *de novo* methylation process. Understanding the process is important to develop strategies for lung cancer prevention, molecular classification, and targeted therapy.

DNMT3B, an important member of DNMT3 family, is a *de novo* methyltransferase, which adds the first methyl group to the cytosine of unmethylated DNA (1, 11, 12). It has been shown that DNMT3B is overexpressed in transformed cells and in multiple types of primary tumors, including NSCLC (13–15). However, the correlation between the expression levels of DNMT3B and promoter methylation status in human cancers has been weak (15–17), suggesting that other key factors are involved in regulation of the promoter methylation. We recently identified a new subfamily of *DNMT3B*, termed *ΔDNMT3B*, due to its lack of part of the NH_2-terminal sequence (18). We showed that *ΔDNMT3B* is the major transcript of *DNMT3B* in NSCLC and has at least seven transcriptional variants resulting from alternative splicing (18). The purpose of the study was to analyze the expression patterns of *ΔDNMT3B* variants in primary NSCLC and to determine their potential relationship with methylation status of tumor suppressor genes commonly altered in NSCLC. To determine biological significance of the molecules, the relationship between expression of the *ΔDNMT3B* variants and patients' clinical outcomes was also analyzed.

Materials and Methods

Patients and specimens. One hundred and nineteen primary tumor samples and their corresponding nonmalignant lung tissues were obtained from patients with stages I to IIIa NSCLC (all stages are pathology stages). All the patients were treated by surgery with curative intend, except those with stage IIIa tumors who might also received postoperative radiation therapy and adjuvant chemotherapy in M. D. Anderson Cancer Center from 1995 to 2000. Samples were immediately frozen and stored at −80°C until analysis. The selection of these patients was based on the availability of archived fresh tumor and corresponding normal lung tissues for the investigators. The clinical information and follow-up data were based on chart review and reports from tumor registry service. Informed consent for the use of residual resected tissues for research was obtained from all the patients enrolled in the study. The study was reviewed and approved by the institution's Surveillance Committee to use the tissues and clinical information. The patients ranged in age from 32 to 84 years (median, 64 years). There were 40 patients with stage I disease, 30 stage II, and 49 stage IIIa. Histologic subtypes include 60 adenocarcinomas, 49 squamous cell carcinoma (SCC), and 7 large cell carcinoma (Table 1). None of the patients had received chemotherapy or radiation treatment before surgery. The median follow-up time was 50.96 months.

RNA extraction and reverse transcription-PCR. Total RNA from tissue samples was extracted by using Tri-Reagent according to the manufacturer's instruction. Approximately 1 to 2 μg of total RNA from each sample

Requests for reprints: Li Mao, Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas M.D. Anderson Cancer Center, P.O. Box 301402, Houston, TX 77030. Phone: 713-792-6363; Fax: 713-796-8655; E-mail: lmao@mdanderson.org.

doi:10.1158/0008-5472.CAN-06-2031

Table 1. Expression of *ΔDNMT3Bs* and clinical/pathological parameters

ΔDNMT3B	*ΔDNMT3B1*, *n* (%)	*ΔDNMT3B2*, *n* (%)	*ΔDNMT3B3*, *n* (%)	*ΔDNMT3B4*, *n* (%)	*ΔDNMT3B5*, *n* (%)	*ΔDNMT3B6*, *n* (%)	*ΔDNMT3B7*, *n* (%)
Sex (*n*)							
F (47)	26 (55.3)	32 (68.1)	0 (0)	16 (34.0)*	9 (19.2)	9 (19.2)	4 (8.5)
M (72)	48 (66.7)	59 (81.9)	3 (4.2)	39 (54.2)	12 (16.7)	23 (31.9)	15 (20.8)
Smoke (*n*)							
No (38)	24 (63.2)	26 (68.4)	1 (2.6)	16 (42.1)	7 (18.4)	9 (23.7)	5 (13.2)
Yes (81)	50 (61.7)	65 (80.3)	2 (2.5)	39 (48.2)	14 (17.3)	23 (28.4)	14 (17.3)
Pathology (*n*)							
Adenocarcinoma (60)	36 (60.0)	45 (75.0)	1 (1.7)	25 (41.7)	9 (15.0)	13 (21.7)	5 (8.3)
SCC (49)	29 (59.2)	37 (75.5)	1 (2.0)	25 (51.0)	10 (20.4)	16 (32.7)	11 (22.5)
Differentiation (*n*)							
Well (11)	6 (54.6)	7 (63.6)	0 (0)	4 (36.4)	0 (0)†	0 (0)†	0 (0)†
Moderate (50)	32 (64.0)	39 (78.0)	1 (2.0)	19 (38.0)	7 (14.0)	11 (22.0)	7 (14.0)
Poor (58)	36 (62.1)	45 (77.6)	2 (3.5)	32 (55.2)	14 (24.1)	21 (36.2)	12 (20.7)
Stage (*n*)							
I and II (70)	43 (61.4)	55 (78.6)	0 (0)	34 (48.6)	11 (15.7)	16 (22.9)	6 (8.6)‡
III (49)	31 (63.3)	36 (73.5)	3 (6.1)	21 (42.9)	10 (20.4)	16 (32.7)	13 (26.5)

*No statistical significant correlation was found, except $P = 0.03$.
†No statistical significant correlation was found, except $P < 0.05$.
‡No statistical significant correlation was found, except $P = 0.009$.

were used to conduct reverse transcription reaction in a 20 μL volume by using SuperScript II RNase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The PCR was carried out in a 12.5 μL volume containing 0.5 μL reverse transcription product, 1.5 mmol/L deoxynucleotide triphosphate (dNTP), 7% DMSO, 6.7 μmol/L $MgCl_2$, 16.6 mmol/L $(NH_4)SO_4$, 67 mmol/L Tris, 10 mmol/L B-mercaptoethanol, 6.7 μmol/L EDTA, 0.5 μmol/L of both the sense and antisense primers, and 0.625 unit of HotStar Taq DNA polymerase (Life Technologies). Amplification was done with an initial denaturing step at 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute in a thermal cycler with a last extension step of 72°C for 10 minutes. PCR products were separated on 2.5% agarose gels and visualized after staining with ethidium bromide. The expression of specific *ΔDNMT3B* variants was determined by using specific primer sets corresponding to unique sequences of individual *ΔDNMT3B* variants as reported previously (18). Any expression observed for a given variant was considered expression positive for both normal and tumor tissues.

DNA extraction and methylation-specific PCR. Frozen tissues were homogenized, and genomic DNA was extracted by digestion of homogenized tissues in buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1% SDS, and 0.5 mg/mL proteinase K at 42°C for 36 hours. The digested products were purified with phenol-chloroform twice. DNA was then precipitated using the ethanol precipitation method and recovered in distilled DNase-free water. For methylation-specific PCR, 1 μg of genomic DNA from each tissue sample was used in the initial step of chemical modification. Briefly, DNA was denatured by NaOH and treated with sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification with the use of Wizard DNA purification resin (Promega Corp., Madison, WI), the DNA was treated again with NaOH. After precipitation, DNA was recovered in water and was ready for PCR with the use of specific primers for either the methylated or the unmethylated *p16* or *RASSF1A* promoter as reported previously (10). PCR was carried out in 25 μL containing ~100 ng of modified DNA, 3% DMSO, all four dNTPs (each at 200 μmol/L), 1.5 mmol/L $MgCl_2$, 0.4 μmol/L PCR primers, and 1.25 units of Taq DNA polymerase (Life Technologies). DNA was amplified for 35 cycles at 95°C for 30 seconds, 60°C for 60 seconds, and 70°C for 60 seconds followed by a 5-minute extension at 70°C in a temperature cycler (Hybaid; Omnigene, Woodbridge, NJ) in 500 μL plastic tubes. PCR products were separated on 2.5% agarose gels and visualized after staining with ethidium bromide. For each DNA sample, primer sets for methylated DNA and unmethylated DNA were used for analysis. CpGenome universal methylated DNA (Chemicon International, Temecula, CA) was used as positive controls, and water replacing for DNA was used as blank controls. The hypermethylation status was determined by visualizing a 150-bp PCR product for *p16* and a 169-bp PCR product for *RASSF1A* with the respective methylation-specific primer sets.

Statistical analysis. The χ^2 test or Fisher's exact test was used to test the association between categorical variables. Overall survival, disease-specific survival (i.e., survival rates among people who died of lung cancer–related causes specifically), and disease-free survival (i.e., recurrence, metastasis, or cancer death is considered as an event) were analyzed. Survival probability was estimated using the Kaplan-Meier method. The log-rank test was used to compare patients' survival time between or among groups. Cox regression was used to model the risks of biological variables on survival time, with adjustment for clinical and histopathologic variables (age, sex, tumor histology subgroup, tumor size, smoking status, and adjuvant treatment). All statistical tests are two sided, and $P < 0.05$ was considered statistically significant.

Results

Expression of *ΔDNMT3B* variants in primary NSCLC and the corresponding normal lungs. Expression of *ΔDNMT3B* variants was detected in 94 of 119 (80%) NSCLCs but in only 22 (20%) of the corresponding normal lungs. The difference of the expression rates between the tumor tissues and normal tissues was statistically significant ($P < 0.0001$). In the tumor tissues, the most frequently expressed variant was *ΔDNMT3B2* (91 or 76%) followed by *ΔDNMT3B1* (74 or 62%) and *ΔDNMT3B4* (55 or 46%) in the 119 primary NSCLCs. Expression of the other variants was less frequent; 32 (27%), 21 (18%), 19 (16%), and 3 (2.5%) for *ΔDNMT3B6, ΔDNMT3B5, ΔDNMT3B7*, and *ΔDNMT3B3*, respectively. Expression of the later group of *ΔDNMT3B* variants was not detected in any of the normal lung tissues.

Correlation between expression of *ΔDNMT3B* variants and clinicopathologic variables. We analyzed correlations between the expression of *ΔDNMT3B* variants and clinicopathologic variables of the patients (Table 1). Tumors from female patients had a higher frequency of *ΔDNMT3B4* expression than tumors from male patients ($P = 0.03$). Expression of *ΔDNMT3B5*, *ΔDNMT3B6*, and *ΔDNMT3B7* was more frequent in poorly differentiated tumors than in well-differentiated or moderately differentiated tumors ($P < 0.05$). The expression of *ΔDNMT3B7* was more frequent in stage III tumors than in stage I/II tumors ($P = 0.009$). No other correlation was observed (Table 1).

Correlation between expression of *ΔDNMT3B* variants and promoter methylation of *p16* and *RASSF1A* genes. Promoter methylation of *p16* and *RASSF1A* was detected in 58 (49%) and 46 (39%) of 119 tumors, respectively. Among the 94 tumors that expressed any of the *ΔDNMT3B* variants, 54 (57%) had *p16* promoter methylation and 46 (48%) had *RASSF1A* promoter methylation compared with 4 of 25 (16%) tumors without expression of any of the variants for *p16* and none for *RASSF1A* ($P < 0.0001$). We then analyzed the relationship between methylation status of *p16* and *RASSF1A* and expression of individual *ΔDNMT3B* variants in the tumor tissues. Promoter methylation of *p16* was correlated with expression of *ΔDNMT3B1*, *ΔDNMT3B2*, *ΔDNMT3B5*, and *ΔDNMT3B6*, whereas promoter methylation of *RASSF1A* was correlated with all the *ΔDNMT3B* variants, except *DNMT3B3* (Table 2). The most striking correlation was between expression of *ΔDNMT3B4* and promoter methylation of *RASSF1A*. Among the 46 tumors with promoter methylation of *RASSF1A*, 42 (91%) expressed *ΔDNMT3B4* compared with only 13 of 73 (18%) tumors without promoter methylation of *RASSF1A* ($P < 0.0001$). In contrast, expression of *ΔDNMT3B4* was not correlated with promoter methylation of *p16* ($P = 0.12$).

Interestingly, promoter methylation of *p16* was associated with promoter methylation of *RASSF1A* ($P = 0.004$). To determine independent factors correlated with promoter methylation, we did multivariate analysis. Results showed that promoter methylation of *p16* and expression of *ΔDNMT3B4* or *ΔDNMT3B7* were independent factors for promoter methylation of *RASSF1A*. After adjusting for *ΔDNMT3B7* expression and promoter methylation of *p16* using logistic regression analysis, expression of *DNMT3B4* remained its strong correlation with promoter methylation of *RASSF1A* ($P < 0.0001$), suggesting that *ΔDNMT3B4* is required for promoter methylation of *RASSF1A* in NSCLC.

Correlation between expression of *ΔDNMT3B* variants and clinical outcomes. We analyzed a potential correlation between expression of *ΔDNMT3B* variants and patients' clinical outcomes. Because many of the patients with stage III tumors underwent postsurgery radiation therapy or chemoradiation therapy whereas patients with stage I/II tumors did not, we analyzed the two groups separately. For stage I/II tumors, patients whose tumors expressed any of *ΔDNMT3B5*, *ΔDNMT3B6*, and *ΔDNMT3B7* had statistically significant poorer overall, disease-specific, and disease-free survivals than patients whose tumors had no expression of *ΔDNMT3B5*, *ΔDNMT3B6*, and *ΔDNMT3B7* ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively; Fig. 1). Because *p16* promoter methylation was statistically correlated with the survivals in the patient population

Table 2. Expression of *ΔDNMT3Bs* and promoter methylation of p16/RASSF1A in NSCLC

ΔDNMT3B		*ΔDNMT3B1*, *n* (%)	*ΔDNMT3B2*, *n* (%)	*ΔDNMT3B3*, *n* (%)	*ΔDNMT3B4*, *n* (%)	*ΔDNMT3B5*, *n* (%)	*ΔDNMT3B6*, *n* (%)	*ΔDNMT3B7*, *n* (%)	*p16*, *n* (%)
ΔDNMT3B2	0	1 (3.6)							
	1	73 (80.2)							
	P	<0.0001							
ΔDNMT3B3	0	71 (61.2)	88 (75.9)						
	1	3 (100.0)	3 (100.0)						
	P	0.29	1						
ΔDNMT3B4	0	28 (43.8)	39 (60.9)	1 (1.6)					
	1	46 (83.6)	52 (94.6)	2 (3.6)					
	P	<0.0001	<0.0001	0.60					
ΔDNMT3B5	0	56 (57.1)	70 (71.4)	3 (100)	37 (37.8)				
	1	18 (85.7)	21 (100)	0 (0)	18 (85.7)				
	P	0.01	0.003	1	<0.0001				
ΔDNMT3B6	0	46 (52.9)	59 (67.8)	1 (1.2)	29 (33.3)	1 (1.2)			
	1	28 (88.5)	32 (100)	2 (6.3)	26 (81.3)	20 (62.5)			
	P	0.0005	<0.0001	0.18	<0.0001	<0.0001			
ΔDNMT3B7	0	57 (57.0)	73 (73.0)	1 (1.0)	39 (39.0)	10 (10.0)	15 (15.0)		
	1	17 (89.5)	18 (94.7)	2 (10.5)	16 (84.2)	11 (57.9)	17 (89.5)		
	P	0.009	0.04	0.07	0.0003	<0.0001	<0.0001		
p16	0	31 (50.8)	40 (65.6)	3 (100)	24 (39.3)	6 (9.8)	10 (16.4)	9 (14.8)	
	1	43 (74.1)	51 (87.9)	0 (0)	31 (53.5)	15 (25.9)	22 (37.9)	10 (17.2)	
	P	0.009	0.004	0.24	0.12	0.02	0.008	0.71	
RASSF1A	0	836 (49.3)	48 (65.8)	1 (1.4)	13 (17.8)	3 (4.1)	8 (11.0)	3 (4.1)	28 (38.4)
	1	38 (82.6)	43 (93.5)	2 (4.4)	42 (91.3)	18 (39.1)	24 (52.2)	16 (34.8)	30 (65.2)
	P	0.0003	0.0005	0.56	<0.0001	<0.0001	<0.0001	<0.0001	0.004

Abbreviations: 0, not expressed for *ΔDNMT3Bs* or not methylated for *p16/RASSF1A*; 1, expressed for *ΔDNMT3Bs* or methylated for *p16/RASSF1A*.

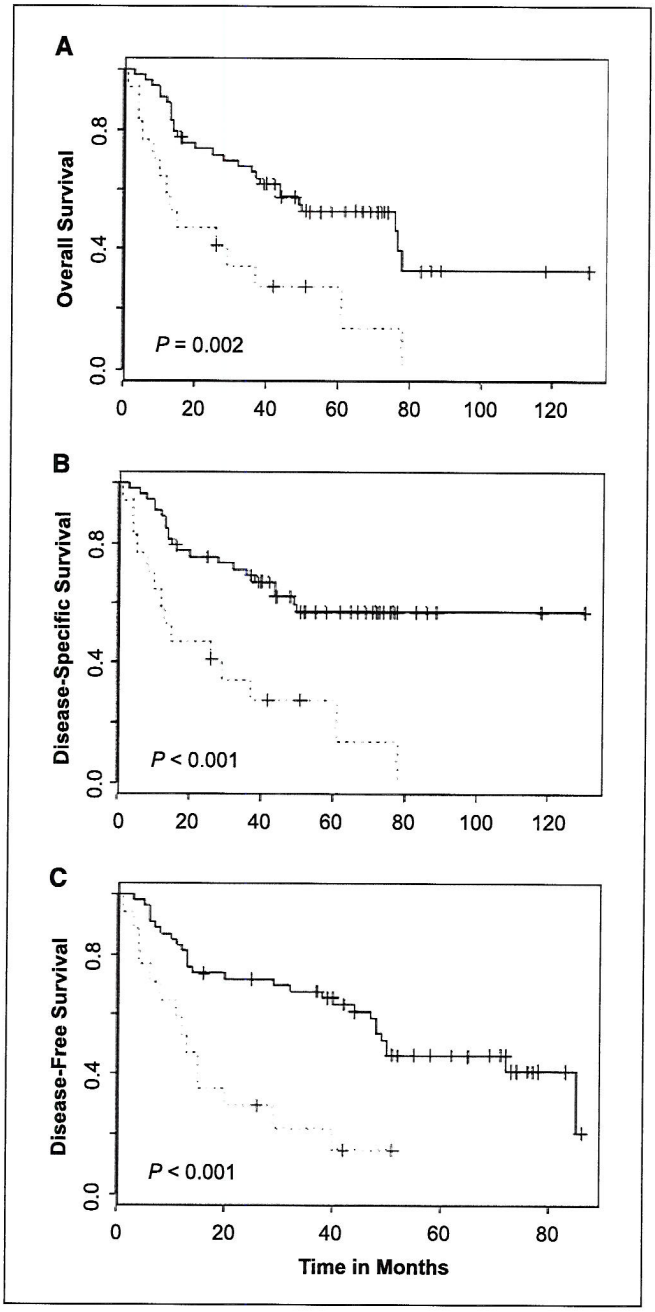


Figure 1. Correlation between expression of any of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* and survivals in patients with stage I/II tumors. *A* to *C*, overall, disease-specific, and disease-free survivals, respectively. *Solid lines*, survival curves of patients whose tumors lacked expression of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7*; *dash lines*, survival curves of patients whose tumors expressed any of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7*.

(10), we did multivariate analysis to correct the confounding factor. Both expression of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* and promoter methylation of *p16* were independent factors in predicting poorer clinical outcomes ($P < 0.01$ for both overall and disease-specific survivals). For stage III tumors, patients whose tumors expressed any of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* variants had poorer survivals ($P < 0.001$; Fig. 2*A-C*) similar to the observation in patients with stage I/II tumors. The difference in this group of patients was that expression of *ΔDNMT3B4* was also strongly correlated with poorer survivals ($P < 0.0001$, $P < 0.0001$, and $P < 0.001$, for overall, disease-specific, and disease-free survivals, respectively; Fig. 2*D-F*). In our previous study, we found that both *p16* and *RASSF1A* promoter methylations were correlated with survivals in the patient population (10). However, in the multivariate analysis, only *ΔDNMT3B4* expression and *p16* promoter methylation were independent factors ($P < 0.001$ for both overall and disease-specific survivals).

Discussion

Overexpression of *DNMT3B*, but not *DNMT1* and *DNMT3A*, has been found common in multiple cancer types, including lung cancer (13, 14, 17), suggesting that *DNMT3B* plays an important role in the development of aberrant promoter methylation during tumorigenesis. However, the correlation between expression levels of *DNMT3B* and promoter methylation status was not strong in human tumors in most reports (15–17). Several studies have suggested that *DNMT3B* alone has limited effect in promoter methylation because the maintenance of methylated promoters of tumor suppressor genes could only be effectively disrupted when both *DNMT3B* and *DNMT1* genes were knocked out, whereas the single knockout of either *DNMT3B* or *DNMT1* had minimal effects (19–21). However, these studies did not address potential effects of individual variants of *DNMT3B*. A dominant-negative effect of *DNMT3b4* by competing with *DNMT3b3* has been suggested, which resulted in DNA hypomethylation on pericentromeric satellite regions (14). This result suggests a complex role of *DNMT3B* variants in regulation of DNA methylation formation. The identification of *ΔDNMT3B*s as the predominant expressing forms of *DNMT3B* in lung cancer (18) further exemplified the complexity of the regulation in lung tumorigenesis.

In this study, we provided a comprehensive view of the expression profiles of the seven *ΔDNMT3B* variants in a large panel of NSCLC tumors and their corresponding normal lungs. The fact that *ΔDNMT3B* variants are frequently expressed in the primary NSCLC but less frequently in the corresponding normal lungs underscores the importance of these molecules in lung tumorigenesis. More importantly, our study provides first *in vivo* evidence to support the importance of individual *ΔDNMT3B* variants in the development of promoter methylation of a particular gene in lung tumorigenesis. We conclude that expression of *ΔDNMT3B4* may contribute to the development of *RASSF1A* promoter methylation. We found that 91% of the NSCLC tumors with methylated *RASSF1A* promoter expressed the variant compared with only 18% of the tumors without *RASSF1A* promoter methylation ($P < 0.0001$). As a comparison, 66% of the tumors without *RASSF1A* promoter methylation expressed *ΔDNMT3B2*, although 94% of the tumors with methylated *RASSF1A* promoter expressed *ΔDNMT3B2* (Table 2). Interestingly, all four tumors with methylated *RASSF1A* but no expression of *ΔDNMT3B4* expressed *ΔDNMT3B2*, suggesting a role of *ΔDNMT3B2* in *RASSF1A* promoter methylation in some NSCLC tumors. To support this notion, none of the 25 tumors that expressed neither *ΔDNMT3B4* nor *ΔDNMT3B2* had methylated *RASSF1A* promoter, whereas 6 of 25 (24%) tumors carried methylated *p16* promoter ($P = 0.02$). Because only two promoters were analyzed in this study, whether *ΔDNMT3B4* is involved in promoter methylation of other genes remains to be determined.

Although the mechanism of differential promoter methylation in tumors is unclear, it is possible that the expression of DNMT variants or the relative expression levels of the variants, including those derived from *DNMT3A* and *DNMT3B*, may, at least in part,

determine patterns of the promoter methylation. One possible explanation of the regulation might be a differential DNA binding through variable PWWP structure, which locates at the more proximal part of DNMT3Bs and is capable to bind DNA directly (22). It is also possible that different variants might have different protein-protein binding capability (23), resulting in different modification of chromatin structures. In either case, different promoters with distinct DNA structures, protein complexes, or protein modifications might be preferentially targeted by individual DNMT variants, resulting in a selected promoter methylation. It should be noted, however, that we only analyzed the *ΔDNMT3B* variants based on the alternative splicing of more proximal exons; therefore, whether the catalytic domains of *ΔDNMT3B4/ΔDNMT3B2* play a role in the process is not addressed in this study.

The association between the expression of certain *ΔDNMT3B* variants and clinical outcomes provides further support for the role

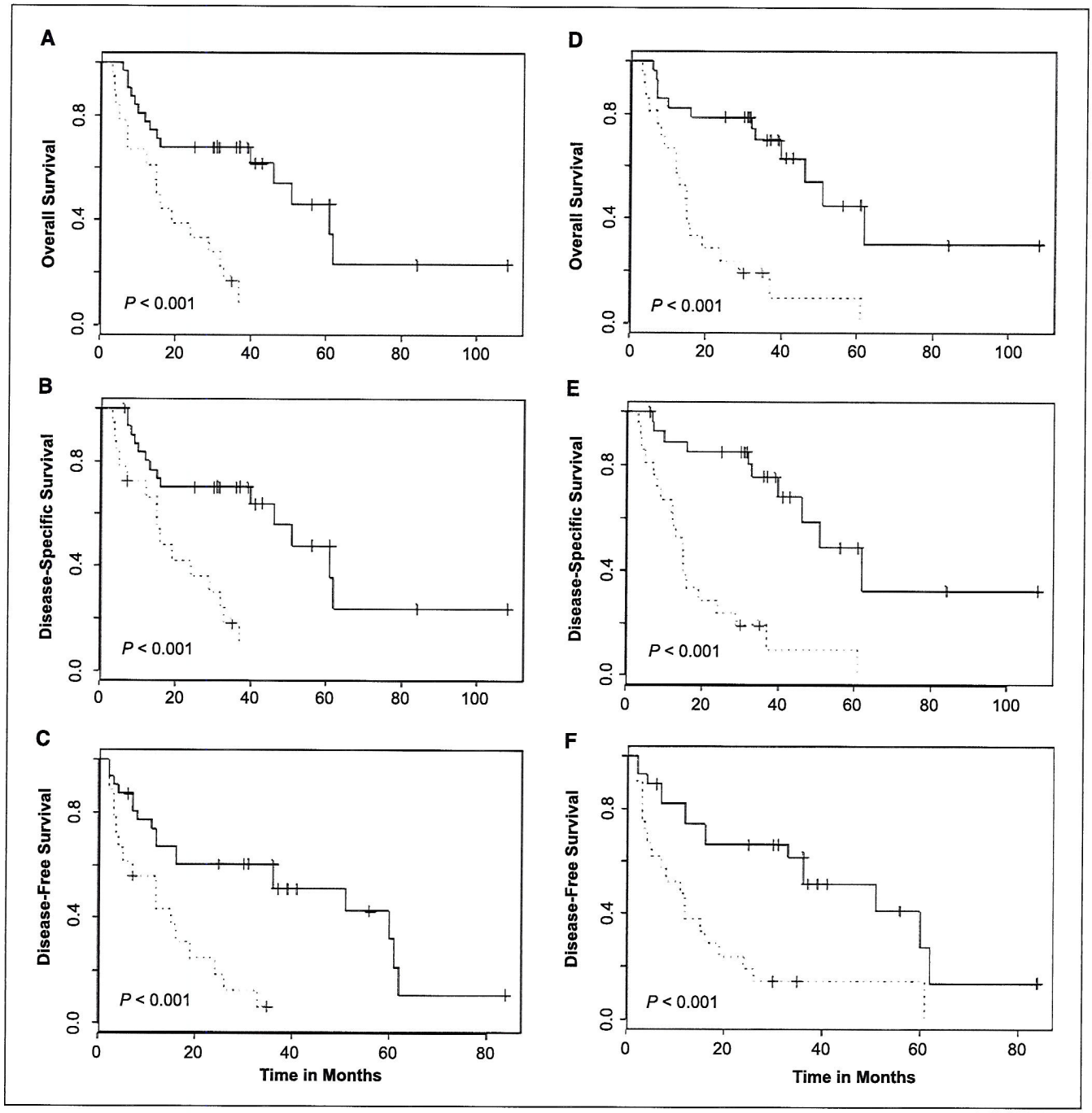


Figure 2. Correlation between expression of *ΔDNMT3B*s and survivals in patients with stage III tumors. *A* to *C*, survivals of patients whose tumors expressed any of *ΔDNMT3B5*, *ΔDNMT3B6*, and *ΔDNMT3B7* (*dash lines*) or had no expression of the variants (*solid lines*) for overall, disease-specific, and disease-free survivals, respectively. *D* to *F*, survivals of patients whose tumors expressed *ΔDNMT3B4* (*dash lines*) or had no expression of *ΔDNMT3B4* (*solid lines*) for overall, disease-specific, and disease-free survivals, respectively.

of *ΔDNMT3Bs* in NSCLC. In patients with either early-stage or locally advanced-stage tumors, the expression of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* correlated with poorer survivals, which was independent of other clinicopathologic variables and promoter methylation status of *p16* and *RASSF1A*. Although the frequencies of *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* expression were relatively low in NSCLC (Table 2), the expression was never detected in the nonmalignant lung tissues, suggesting that *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* are involved in the late-stage tumorigenesis of lungs. It is interesting to note that the predicted proteins from *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7* contain no enzymatic domain of the methyltransferase due to premature translational termination (18). Unfortunately, the lack of high-quality antibodies for the variants prevents us to evaluate protein expression of the variants in the tumors at this time. Whether their biological roles are inserted through their variable COOH-terminal regions remains to be determined.

In summary, we did the first comprehensive analysis to determine the expression profiles of *ΔDNMT3B* variants in large number of primary NSCLC tumors and their corresponding normal lung tissues. We revealed a strong correlation between the expression of *ΔDNMT3B4* and *RASSF1A* promoter methylation, suggesting a role of the variant in regulation of promoter methylation during lung tumorigenesis. We also found that expression of certain *ΔDNMT3B* variants (i.e., *ΔDNMT3B5, ΔDNMT3B6*, and *ΔDNMT3B7*) was correlated with poor clinical outcomes, suggesting their role in NSCLC progression.

Acknowledgments

Received 6/2/2006; revised 7/12/2006; accepted 7/20/2006.

Grant support: Department of Defense grant DAMD17-01-1-01689-1.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases DNMT3a and DNMT3b are essential for *de novo* methylation and mammalian development. Cell 1999;99:247–57.
2. Jaenisch R. DNA methylation and imprinting: why bother? Trends Genet 1997;13:323–9.
3. Kass SU, Pruss AP, Wolffe AP. How does DNA methylation repress transcription? Trends Genet 1997; 13:444–51.
4. Surani MA. Imprinting and the initiation of gene silencing in the germline. Cell 1998;93:309–16.
5. Robertson KD, Wolffe AP. DNA methylation in health and disease. Nat Rev Genet 2000;1:11–9.
6. Jones P, Gonzalgo M. Altered DNA methylation and genome instability: a new pathway to cancer? Proc Natl Acad Sci U S A 1997;94:2103–5.
7. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 2001;61:249–55.
8. Safar AM, Spencer H III, Su X, et al. Methylation profiling of archived non-small cell lung cancer: a promising prognostic system. Clin Cancer Res 2005;11: 4400–5.
9. Tang X, Khuri FR, Lee JJ, et al. Hypermethylation of the DAP-kinase CpG island correlates with aggressive biological behavior in stage I non-small cell lung cancer. J Natl Cancer Inst 2000;92:1511–6.
10. Wang J, Lee JJ, Wang L, et al. Distinct value of $p16^{INK4a}$ and *RASSF1A* promoter methylation in prognosis of the patients with respectable non-small cell lung cancer. Clin Cancer Res 2004;9:4415–22.
11. Aoki A, Suetake I, Miyagawa J, et al. Enzymatic properties of *de novo*-type mouse DNA(cytosine-5) methyltransferases. Nucleic Acids Res 2001;29: 3506–12.
12. Xie S, Wang Z, Li E. Cloning, expression, and chromosome locations of the human DNMT3 gene family. Gene 1999;236:87–95.
13. Robertson KD, Uzvolgyi E, Liang G, et al. The human DNA methyltransferases (DNMTs) 1, 3a, and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 1999;27: 2291–8.
14. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U S A 2002;99:10060–5.
15. Eads CA, Kathleen DD, Kazuyuki K, Leonard BS, Peter VD, Peter WL. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res 1999;59: 2302–6.
16. Sato M, Horio Y, Sekido Y, Minna JD, Shimokata K, Hasegawa Y. The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a), and RASSF1A in human lung cancer cell lines. Oncogene 2002;21:4822–9.
17. Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 2003;9: 4415–22.
18. Wang L, Wang J, Sun S, et al. A novel DNMT3B subfamily, ΔDNMT3B, is the predominant form of DNMT3B in non-small cell lung cancer. Int J Oncol 2006;29:201–7.
19. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002;416:552–6.
20. Rhee I, Jair KW, Yen RW, et al. CpG methylation is maintained in human cancer cells lacking DNMT1. Nature 2000;404:1003–7.
21. Leu YW, Rahmatpanah F, Shi H, et al. Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 2003; 63:6110–5.
22. Qiu C, Sawada K, Zhang X, Cheng X. The PWWP domain of mammalian DNA methyltransferase Dnmt3b defines a new family of DNA-binding folds. Nat Struct Biol 2002;9:217–24.
23. Ge YZ, Pu MT, Gowher H, et al. Chromatin targeting of *de novo* DNA methyltransferases by the PWWP domain. J Biol Chem 2004;279:25447–54.

A novel *DNMT3B* subfamily, *ΔDNMT3B*, is the predominant form of *DNMT3B* in non-small cell lung cancer

LUO WANG[1], JIE WANG[5], SHIYONG SUN[3], MARIVONNE RODRIGUEZ[2], PING YUE[3], SE JIN JANG[4] and LI MAO[1]

[1]Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, [2]Department of Molecular Genetics, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030; [3]Department of Oncology, Winship Cancer Institute, Emory University, Atlanta, GA, USA; [4]Department of Pathology, Hanyang University, Kuri Hospital, 471-701 Seoul, Korea; [5]Department of Thoracic Medical Oncology, Peking University School of Oncology and Beijing Cancer Hospital, Beijing, P.R. China

Received February 3, 2006; Accepted February 27, 2006

Abstract. *De novo* promoter DNA methylation represses gene transcription and is a common mechanism to inactivate tumor suppressor genes in tumorigenesis. *DNMT3B* plays an important role in *de novo* DNA methylation. We report here the identification of a novel *DNMT3B* subfamily, termed *ΔDNMT3B*, whose expression is initiated through a promoter located at intron 4 and exon 5 of the *DNMT3B* gene. At least 7 transcriptional variants of *ΔDNMT3B* have been observed as the result of alternative pre-mRNA splicing. Predicted proteins derived from these variants suggest that 4 of the variants share a conservative enzymatic domain but contain a variable PWWP motif, a putative DNA binding structure, whereas 3 of the variants lack the enzymatic domain due to predicted premature translational termination. In non-small cell lung cancer (NSCLC) cell lines, *ΔDNMT3B* variants are frequently expressed and are the predominant forms of *DNMT3B*. Similarly, *ΔDNMT3B* variants are frequently expressed in primary NSCLC but are not detectable or are expressed at low levels in corresponding normal lung tissue. Our results indicate that *ΔDNMT3B* is the major expression form of *DNMT3B* in NSCLC and may play an important role in the development of aberrant promoter methylation during lung tumorigenesis.

Correspondence to: Dr Li Mao, Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
E-mail: lmao@mdanderson.org

Abbreviations: DNMT, DNA methyltransferase; PWWP, proline-tryptophan-tryptophan-proline; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-polymerase chain reaction

Key words: ΔDNMT3B, NSCLC, promoter, expression, splicing

Introduction

DNA methylation plays an essential role in normal development of mammalian embryo by regulating gene transcription through genomic imprinting, X chromosome inactivation, and genomic stability (1-4). It is believed that DNA methylation patterns in somatic cells are established during gametogenesis and early embryonic development via consecutive waves of demethylation and *de novo* methylation (5). *DNMT3* consists of *DNMT3A* and *DNMT3B* and has been shown to be the major *de novo* DNA methyltransferase (6,7) that preferentially methylates cytosine in CpG sites. The methylation in CpG-rich promoter regions would result in transcriptional silencing of the corresponding genes.

Okano *et al* found that murine *Dnmt3a* and *Dnmt3b* are highly expressed in the undifferentiated ES cells but are down-regulated during development and maintained at a low level in somatic cells (6). They further revealed that, at E7.5, *Dnmt3b* was highly expressed in the embryonic ectoderm, neural ectoderm, and chorionic ectoderm while a weak expression was detected in mesodermal and endodermal cells (8). The expression of *Dnmt3a* was moderate in embryonic ectoderm and weak in mesodermal cells until E8.5 and E9.5, at which point *Dnmt3a* expression became ubiquitous with increased expression in the somites and the ventral part of the embryo (8). These observations suggest that the two types of enzymes may function differently in development.

Human *DNMT3B* is highly homologous to the mouse gene and contains 24 exons spanning approximately 47 kb of genomic DNA. Two alternative 5' exons of *DNMT3B*, both resulting in full-length *DNMT3B* (*DNMT3B1* and *DNMT3B2*), have been reported (9). Three transcription variants resulting from alternative splicing have also been reported (*DNMT3B3-5*) (9). Some of the variants lacking DNA methyltransferase activity may compete with variants with enzyme activity resulting in DNA hypomethylation (10), suggesting a complex role of *DNMT3B* variants. Increased expression of *DNMT3B* has been frequently observed in human cancer cell lines and primary tumors compared to most normal tissues except testis,

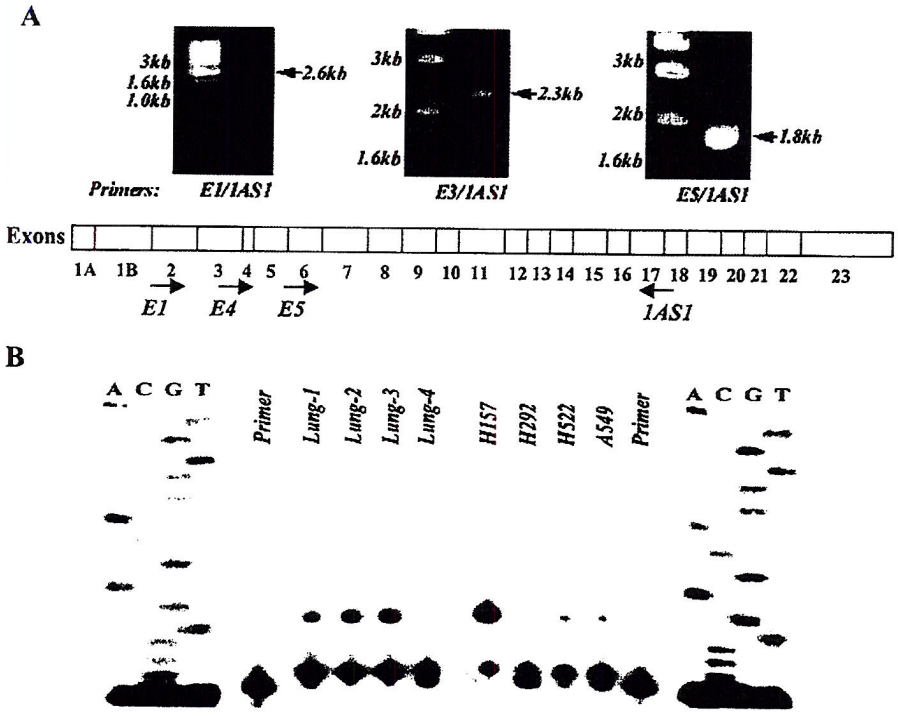


Figure 1. Identification of *ΔDNMT3B*. (A) Expression of the *DNMT3B* 5'-region measured using RT-PCR with different 5' primers located at exon 2 (E1), exon 4 (E2), and exon 6 (E3) of *DNMT3B1*, respectively. (B) Primer extension analysis showing transcription starting points of *ΔDNMT3B*.

pancreas, thyroid, and bone marrow (9-11). However, the level of *DNMT3B* expression in tumors does not consistently correlate with the promoter methylation status of genes (11-13). We report here the identification of a new class of *DNMT3B* transcripts expressed through a novel promoter, termed *ΔDNMT3B*. We further demonstrate that *ΔDNMT3B* is the predominant form of *DNMT3B* in NSCLC, suggesting an important role of *ΔDNMT3B* in DNA methylation control and lung tumorigenesis.

Materials and methods

Cell lines and primary tissues. Human NSCLC lines H157, H226, H292, H460, H522, H1299, H1944, and A549 were purchased from the American Type Cell Culture (Rockville, MD). Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in the presence of 5% CO_2.

Twelve paired primary tumor tissues and corresponding normal lung tissues from patients with primary NSCLC were obtained from surgically resected specimens collected in the Department of Pathology at The University of Texas M.D. Anderson Cancer Center and stored at -80°C until the experiment. The study was approved by the Institutional Review Boards of The University of Texas M.D. Anderson Cancer Center.

RNA extraction and RT-PCR. The total RNA for each cell line and clinic sample was isolated using Tri reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Approximately 1 μg of total RNA from each sample was used to conduct a reverse transcription reaction in a 20-μl volume using SuperScript II RNase H-reverse transcriptase (Gibco BRL Life Technologies Inc., Grand Island, NY). The synthesized cDNA was either used immediately for PCR amplification or stored at -20°C for further analysis.

The mRNA expression levels of total *DNMT3Bs* were detected using a primer set of the forward primer, S1 (5'-GAG TTG GGC ATA AAG GTA GG-3'), and the reverse primer, 1AS1 (5'-TGA GGT ACA CGG TAT GAC C-3'), located at exon 17 and the 3'-untranslation region of *DNMT3B1*, respectively. The 5'-end forward primers, E1 (5'-CAT GAA GGG AGA CAC CAG GC-3'), E3 (5'-ATG CCA AAG CTC TTC CGG GA-3'), and E5 (5'-TGG AGA TGG AGA CAG TTC AG-3'), and the reverse primer, 1AS1, were also used to detect *DNMT3Bs* (Fig. 1A).

PCR was performed in a 12.5-μl volume containing 0.5 μl of reverse transcription products, 7% dimethylsulfoxide, 1.5 mM deoxynucleotides (dNTPs), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris, 10 mM ß-mercaptoethanol, 6.7 μM ethylene diamine tetra acetic acid (EDTA), 0.5 μM of both the forward and the reverse primer, and 0.625 U of HotStar Taq DNA polymerase (Qiagen, Inc., Chatsworth, CA). Amplification was carried out in a thermal cycler (PCR Express, Hybaid, Middlesex, UK) with an initial denaturing step at 95°C for 15 min, followed by 35 cycles at 95°C for 30 sec, 58-62°C for 1 min, and 72°C for 1 min, with a last extension step at 72°C for 10 min. The PCR products were mixed with 6X loading buffer containing 0.5 mg/ml ethidium bromide and separated by electrophoresis on a 2% agarose gel.

Primer extension and nuclease S1 mapping. To determine the exact starting site of the *ΔDNMT3B* transcript, standard primer

extension and S1 mapping methods were used with the [γ-^{32}P]-ATP end-labeled antisense primer, 3B6AS (5'-GGT AGC CGG GAA CTC CAC GG-3'). For primer extension, briefly, 1 μg of total RNA was mixed with [^{32}P]-labelled primer. The mixture was incubated at 70˚C for 15 min and then at room temperature for 10 min. Extension reactions (20 μl) consisted of 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dNTP, and 200 U SuperScript™ II reverse transcriptase (Gibco BRL Life Technologies Inc.). Reactions were incubated at 37˚C for 15 min. The products were mixed with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.02% xylene cyanole FF), denatured at 98˚C for 5 min, and then separated on a 12% acrylamide-7 M urea denatured gel. Radioactive signals were detected by autoradiography.

Nuclease S1 mapping was performed with a 1080-bp DNA fragment which was amplified using the forward primer, E4INT-1 (5'-TGC TGT GAC AGG CAG AGC AG-3'), and the reverse primer, E5AS (5'-TCT GTG TCG TCT GTG AGG TC-3'). After cloning this fragment into a PCR® 2.1-TOPO® vector (Invitrogen Corp., Carlsbad, CA), a 320-bp fragment of single-strand DNA probe used for S1 nuclease mapping was generated by single-primer PCR using [^{33}P]-labelled internal primer 3B6AS. The PCR condition was the same as above. This single-strand 320-bp PCR product was separated in a 2% agarose gel and purified using a QIAquick gel extraction kit (Qiagen Inc.) followed by recovering in 50 μl Tris-buffer (10 mM Tris-HCl, pH 8.5).

The total RNA from different samples was co-precipitated with 50 ng of recovered 320-bp [^{33}P]-labelled probe. Samples were dissolved in 30 μl of hybridization buffer (40 mM MOPS, pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% formamide) and incubated at 85˚C for 15 min. After overnight hybridization at 54˚C based on the GC content of the projected fragment, the samples were digested for 1 h at 37˚C with S1 nuclease (Gibco BRL Life Technologies Inc.) in the buffer containing 30 mM sodium acetate, pH 4.6, 1 mM zinc acetate, 5% glycerol, and 0.28 M NaCl. The resulting products were detected as described in primer extension section.

Construction of DNMT3B6 promoter and luciferase assay. The first 1080-bp Δ*DNMT3B* promoter was amplified with the primer set of E4INT-1 and E5AS. This fragment contains, 355 bp upstream of the Δ*DNMT3B* transcription starting site, the first exon and intron of Δ*DNMT3B*. After inserting the fragment into the pGL3-basic vector (Promega Corp., Madison, WI), the plasmids containing both forward (F) and reverse (R) directions were used for transient transfection. To compare the functional difference of C/T polymorphism in the Δ*DNMT3B* promoter region, both pGL3 T-type and C-type promoters were constructed.

Lung cancer cell line A549 and H157 (ATCC, Manassas, VA) was used for transient transfection using FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's instructions. The plasmid, pCH110 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), was used as the internal control for monitoring the transfection efficiency. The signal was detected using a luciferase assay system (Promega Corp.) in a luminometer (Lumat 9507, EG&G Berthold). The values of luciferase activity were normalized against those of ß-galactosidase expressed by plasmid pCH110.

Detection of individual ΔDNMT3B splicing variants. The expression levels of specific *DNMT3B* and Δ*DNMT3B* variants in NSCLC cell lines and primary tissues were determined using specific primer sets corresponding to individual *DNMT3B* and Δ*DNMT3B* variants. For Δ*DNMT3B1*, we used 1S, 5'-TGG AAG GCC ACC TCC AAG C-3', as the forward primer and 1AS, 5'-GCC TGC ACG ACG CAC CTT CG-3', as the reverse primer; for Δ*DNMT3B2*, 2S 5'-AGA TCA AGG GCT TCT CCT GG-3' and 2AS 5'-GAG TCT TGT TCT CTG GTT GCG-3'; for Δ*DNMT3B3*, 3S 5'-GTT CAG AGT ATC AGG TCT CTG C-3' and 1AS; for Δ*DNMT3B4*, 3S and 2AS; for Δ*DNMT3B5*, 4S 5'-GTT CAG AGT ATC AGA GAA CAA GAC-3', and 3AS 5'-CTG CCA CAA GAC AAA CAG CC-3'; for Δ*DNMT3B6*, 5S 5'-GTT CTC CGA GAG AAC AAG AC-3', and 4AS 5'-CAG TAA GAC TGA TAG CCA TCG-3'; and for Δ*DNMT3B7*, 6S 5'-TGC TCT GGA GAG AAC AAG AC-3', and 5AS 5'-GAG ACA CAT GTA ACA GCT CC-3'. A common forward primer, E1 5'-TGC TAA GCT ACA CAC AGG AC-3', was used for the *DNMT3B* variants, and specific reverse primers were used to distinguish individual variants as follows: 1AS for *DNMT3Bs* corresponding to Δ*DNMT3B1* and Δ*DNMT3B3*; 2AS for *DNMT3Bs* corresponding to Δ*DNMT3B2* and Δ*DNMT3B4*; 6AS, 5'-CGA GTC TTG TTC TCT GAT ACT C-3', for *DNMT3B* corresponding to Δ*DNMT3B5*; 7AS, 5'-CGA GTC TTG TTC TCT CGG AG-3', for *DNMT3B* corresponding to Δ*DNMT3B6*; and 8AS, 5'-CGA GTC TTG TTC TCT CCA G-3', for *DNMT3B* corresponding to Δ*DNMT3B7*.

Results

To determine the expression levels of *DNMT3B1* in normal lung tissue and lung cancer tissue, we analyzed 12 pairs of primary NSCLC tissue and corresponding normal lung tissue using RT-PCR with a set of primers located at exon 17 and exon 23 of *DNMT3B1* respectively. We found that *DNMT3B1* expression was either undetectable or at trace levels in the vast majority of normal lung tissue analyzed while it was detectable in all NSCLC tissue with 50% (6/12) of the tumors expressed at a high level. To validate the finding, we designed several additional sets of primers that allowed us to amplify *DNMT3B1* mRNA at different exon locations closer to its transcriptional initiation site. We found that the expression level of the gene was much lower when using a primer located at exon 2 (E1) or exon 4 (E4) compared to a primer located at exon 6 (E5) of *DNMT3B1* (Fig. 1A), suggesting the presence of additional transcripts excluding exons 2-4. To confirm this observation, we tested other primer sets at these regions and the results were consistent with the previous observation (data not shown). To exclude the possibility of contamination with homologue molecules in the RT-PCR products, we performed direct sequencing analysis of each RT-PCR product. The sequences matched perfectly to the originally reported corresponding transcript sequence of *DNMT3B1* [GenBank accession number (AN): AL035071].

To determine the exact starting point(s) of the novel transcriptions, we performed primer extension assay using RNA

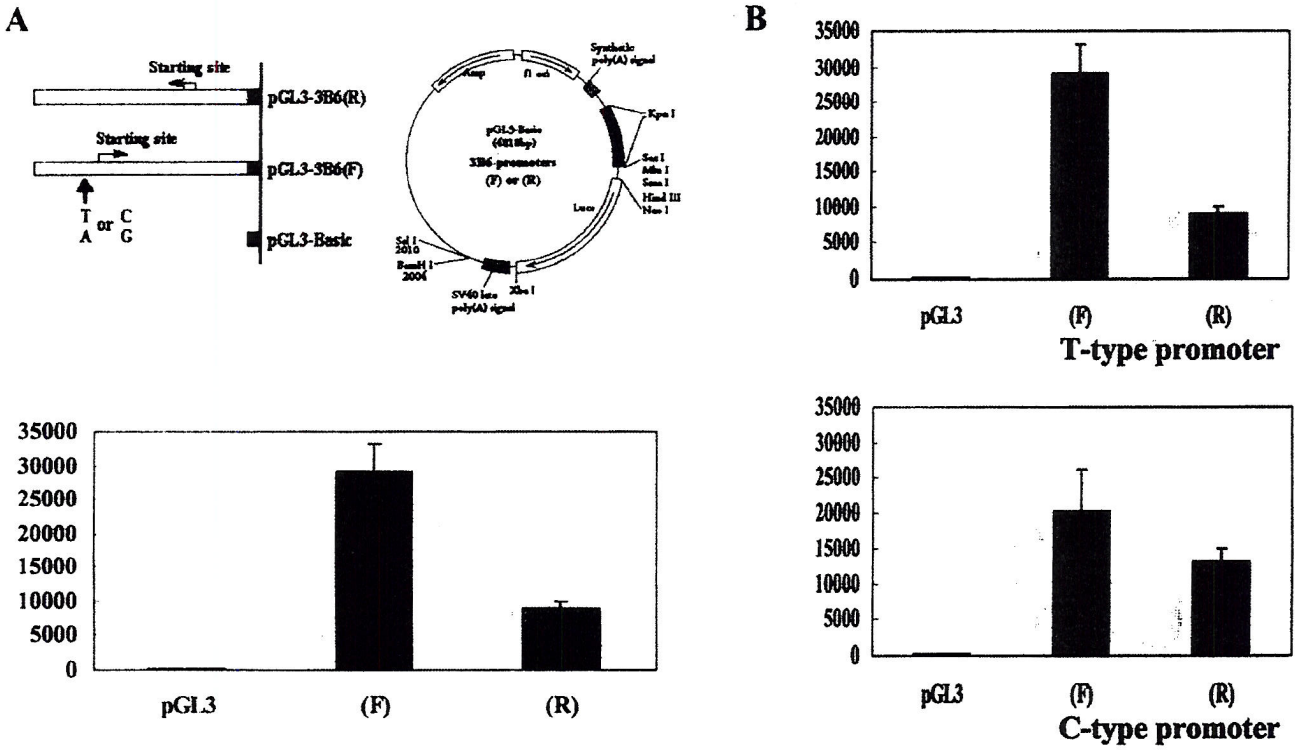


Figure 2. Promoter of *ΔDNMT3B*. (A) Activity of *ΔDNMT3B* promoter detected using luciferase assay. (B) Effect of a T→C transition (polymorphism) on promoter activity of *ΔDNMT3B*. The larger arrow indicates the position of the polymorphism. Based on the polymorphism, the promoters are defined as C-type or T-type. F indicates forward direction of the promoter and R indicates reverse direction of the promoter.

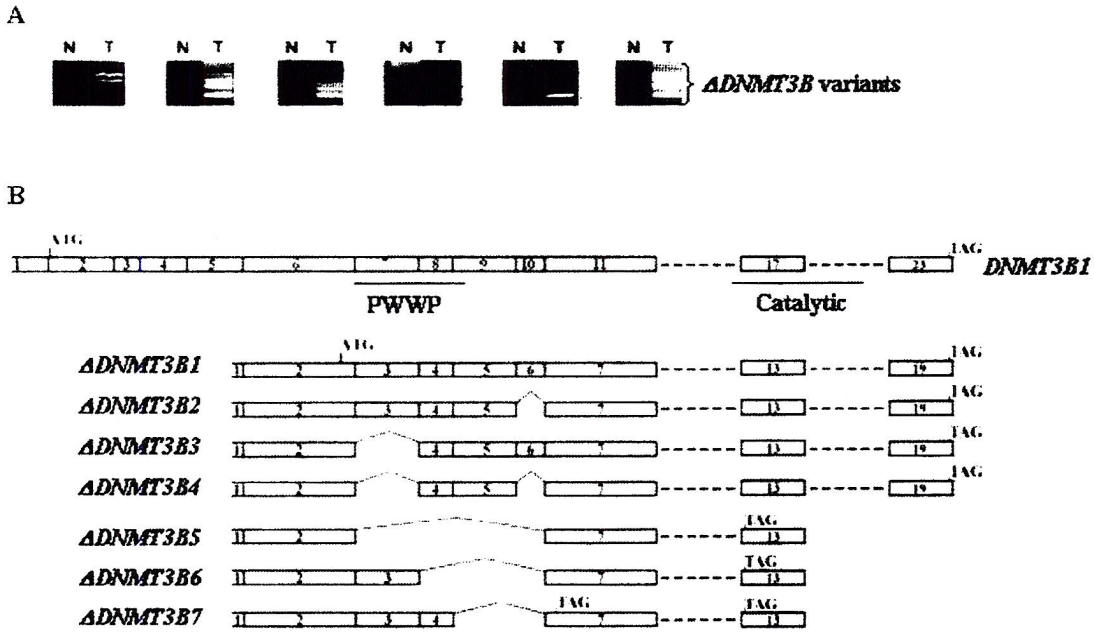


Figure 3. Alternative splicing of *ΔDNMT3B*. (A) High level expression of *ΔDNMT3B* variants in lung cancer tissue compared to corresponding normal lung tissue. (B) The structure scheme of *DNMT3B1* and *ΔDNMT3B* variants. N indicates normal tissue and T indicates cancer tissue.

templates from lung cancer tissue and NSCLC cell lines and a primer (3B6AS) located at exon 5 of *DNMT3B1*. We identified two major transcriptional initiation sites located at nt 23990 and nt 23994 within exon 5 of *DNMT3B1* (GenBank AN: 15306493), respectively (Fig. 1B). This partial exon 5 was then named as the first exon of the novel transcript(s) containing either 28 bp or 24 bp depending on which transcriptional initiation site it derives from. We further validated the finding by using nuclease S1 RNA mapping analysis (data not shown). We designated the new transcript from these

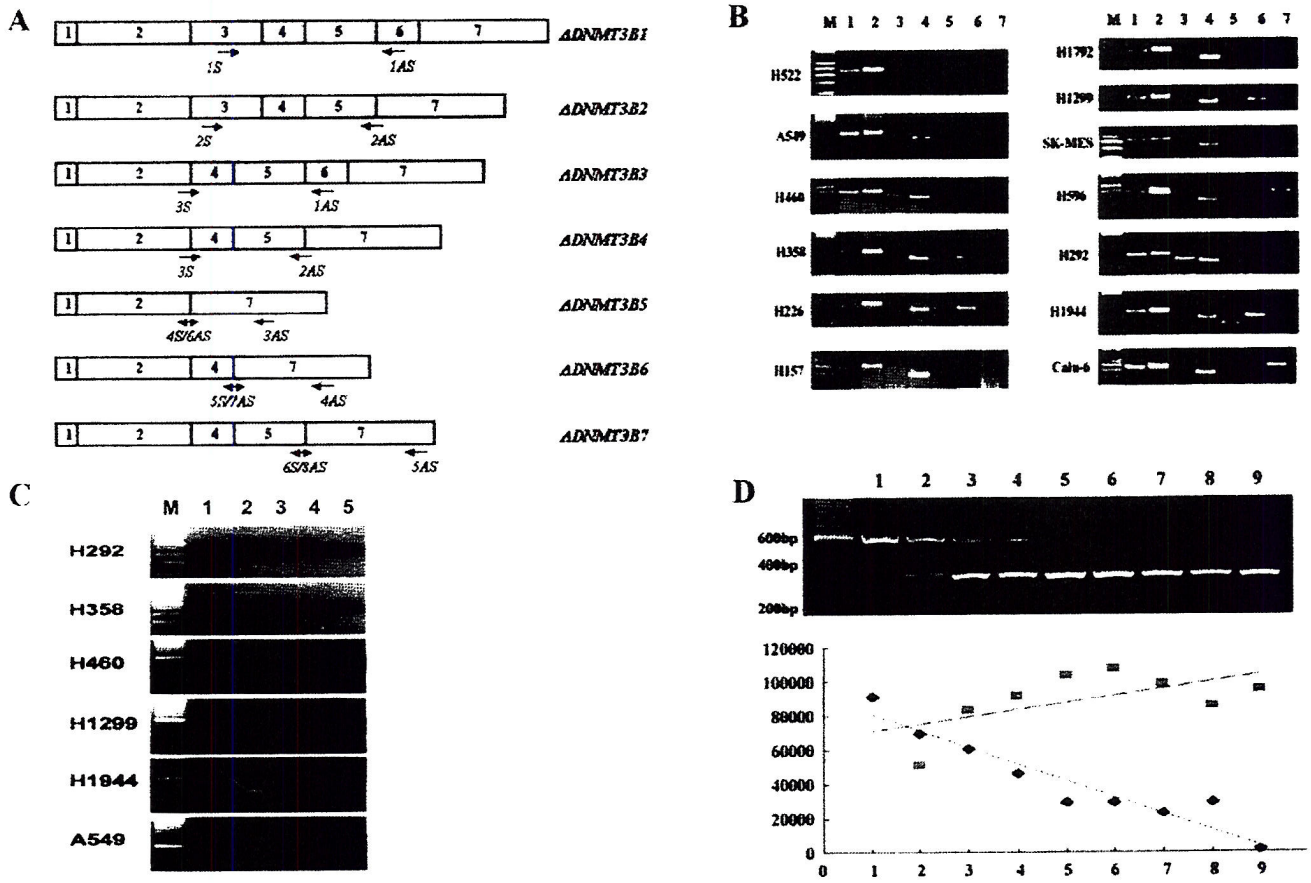


Figure 4. Alternative or aberrant splicing variants of *ΔDNMT3B* subfamily. (A) Location of the primers used to amplify individual *ΔDNMT3B* variants in this study. (B) Expression patterns of *ΔDNMT3B* variants in NSCLC cell lines; 1-7 represent *ΔDNMT3B1-7*, respectively. (C) Expression patterns of *DNMT3Bs*, with more proximal exons corresponding to *ΔDNMT3B1-4* and *ΔDNMT3B6*. (D) Multiplex PCR using primer sets for *DNMT3B1* and *ΔDNMT3B1* with different ratios in concentration (concentration of *DNMT3B1* primer set was serially diluted from 1 to 9 and serially increased from 1 to 9; 1, *DNMT3B1* primer set alone; 9, *ΔDNMT3B1* primer set alone; 5, equal concentrations for both primer sets). The upper band represents the *DNMT3B1* product and the lower band represents the *ΔDNMT3B1* product. The lower panel shows relative intensity of the product bands.

starting sites as *ΔDNMT3B* because it lacks exons 1-4 of *DNMT3B*.

To determine the existence of a potential promoter upstream of the newly identified transcript, we constructed a 1080-bp DNA fragment containing, 355 bp upstream of the *ΔDNMT3B* transcriptional initiation site, the first exon and intron of *ΔDNMT3B* and partial exon 2 into a vector containing a reporter gene. We performed a promoter activity assay and found promoter activity of the DNA fragment (Fig. 2A). We further constructed serial plasmids with both sense and reverse sequences of the DNA fragment and various deletions. Using these constructs, we found that the core promoter activity of *ΔDNMT3B* is in a 477-bp fragment containing one repressor element and three cis-acting elements (data not shown). Interestingly, a common T→C transition polymorphism was found in the promoter region of *ΔDNMT3B* located -286 bp from the transcriptional initiation site, which may change a TFIID (CTcTATTCCA) binding site to GATA-1 (TCTATC) binding site. We noticed a stronger promoter activity with the T form than the C form (18-fold vs. 12-fold compared to the control, respectively) (Fig. 2B).

Because of the presence of various sizes of RT-PCR products using these primer sets, we suspected that *ΔDNMT3B* might contain multiple splicing variants (Fig. 3A). By directly sequencing the individual fragments, we found at least seven variants (by including or excluding different combinations of exons 3, 4, 5, and 6 of *ΔDNMT3B*), which we designated as *ΔDNMT3B1-7* (Fig. 3B). A comparative analysis of the putative amino acid sequences of the variants showed that *ΔDNMT3B* lacked 199 amino acids at the N-terminal, compared with *DNMT3B1*, and that *ΔDNMT3B1* and *ΔDNMT3B2* contained a complete PWWP motif; other variants either contained a partial PWWP motif or no such structure; *ΔDNMT3B5-7* lacks the enzymatic domains because of a premature termination that results from frame shifting after alternative splicing (Fig. 3B).

To detect individual *ΔDNMT3B* variants, we designed specific PCR primer sets on the basis of their splicing patterns (Fig. 4A). We detected *ΔDNMT3B1* and *ΔDNMT3B2* expression in all 13 NSCLC cell lines analyzed, *ΔDNMT3B4* expression in 12 of the 13 cell lines, and *ΔDNMT3B6* expression in 7 of the 13 cell lines; in contrast, expression of *ΔDNMT3B3*, *ΔDNMT3B5*, and *ΔDNMT3B7* were less frequent (Fig. 4B). Interestingly, the *DNMT3B* variants were expressed less frequently and at lower levels in these cell lines (Fig. 4C). In a multiplex PCR analysis, we determined

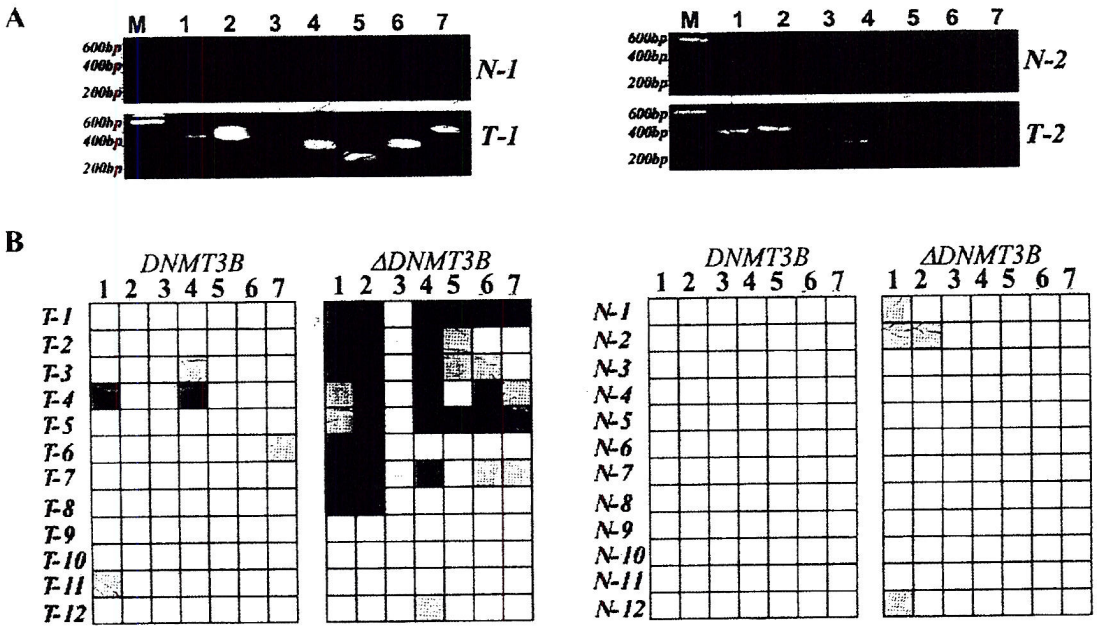


Figure 5. Expression of *ΔDNMT3B* variants in primary lung cancer tissue and corresponding normal lung tissue. (A) Examples of *ΔDNMT3B* variant expression patterns in paired normal lung tissue and primary lung cancer tissue. (B) Expression patterns of *DNMT3B* and *ΔDNMT3B* variants in paired normal (N) lung tissue and primary tumor (T) tissue. The density of the shed boxes represents the relative level of the gene expression.

the relative amplification efficiencies of the primer sets for *DNMT3B* and *ΔDNMT3B* using DNA templates containing various concentrations of *DNMT3B* and *ΔDNMT3B1* (Fig. 4D). The robust amplification of *DNMT3B1* (Fig. 4D) indicated that the lack or very low levels of RT-PCR products in the cell lines reflected the low level of the corresponding *DNMT3B* transcripts.

To determine whether the expression profiles observed in the NSCLC cell lines also exist in the primary NSCLC, we analyzed 12 pairs of primary NSCLC tumors and corresponding normal lung tissue. We found that the expression of *ΔDNMT3Bs* but not *DNMT3Bs* was frequent in the primary tumors whereas the expression of the variants was mainly non-detectable or weakly expressed in the corresponding non-cancerous lungs (Fig. 5). Nine (75%) of the 12 tumors expressed at least one variant, similar to the cancer cell line data.

Discussion

DNA methyltransferases play an important role in initiation and maintenance of cytosine methylation in human genome. Although the role of DNMT1 is primarily to maintain DNA methylation status and the role of DNMT3s relates to *de novo* DNA methylation, recent studies have shown that these molecules not only possess distinct activities (6,8,14) but also interact with each other in complex biological processes to regulate patterns of DNA methylation in human genome (15-18). The dominant-negative effect of *DNMT3b4*, which lacks methyltransferase enzymatic motifs, in competing with *DNMT3b3* to result in DNA hypomethylation on pericentromeric satellite regions (10) suggests an important role of the isoforms of the gene. The identification of *ΔDNMT3B* and its multiple splicing variants in this study further complicate the role of *DNMT3B* family members in regulating DNA methylation in physiological and pathological conditions.

ΔDNMT3B derives from a novel promoter located upstream of exon 5 of *DNMT3B1* with a putative translation initiation site at exon 6 (exon 2 of *ΔDNMT3B*). As a result, the predicted proteins of *ΔDNMT3Bs* lack 200 amino acids at the N-terminal of *DNMT3B1* but maintain the PWWP domain in several variants (*ΔDNMT3B1-4*). Therefore, these putative proteins may share some common function, such as DNA methyltransferase activity, with *DNMT3B* but possess other distinct biological features. Because predicted *ΔDNMT3B5-7* lack the enzymatic domain of DNA methyltransferase, their biochemical functions may be more distinct than those of their other family members.

In previous studies, *DNMT3B* has been found to be more highly expressed in cancer cell lines and primary tumors than in normal tissue; however, an association between the expressional level of *DNMT3B* and promoter methylation of tumor suppressor genes was not established (11-13,19). One possible explanation was that *DNMT3B* expression may be regulated in the cell cycle and that the increased expression observed in tumors is merely a reflection of increased cell proliferation (13). Studies have shown that both *DNMT3B* and *DNMT1* genes are necessary for maintaining the methylated promoters of tumor suppressor genes (20,21). Another study showed that *DNMT3b4*, a *DNMT3B* variant lacking methyltransferase enzymatic motifs, might act as a dominant-negative factor to reduce DNA methylation (10), suggesting that splicing variants of *DNMT3B* may play distinctive roles in regulating DNA methylation. The identification of *ΔDNMT3Bs* in this study adds additional complexity to the current knowledge of *DNMT3B* in biological systems.

The high expression of *ΔDNMT3Bs* but not *DNMT3Bs* in both NSCLC cell lines and primary tumors suggests the

importance of the Δ form in lung tumorigenesis. Because the tumors express different patterns of *ΔDNMT3B* variants, it is possible that the expression of variable *ΔDNMT3Bs* rather than the overall expression levels plays a role in promoter methylation in lung tumorigenesis. Further studies are needed to address this issue.

A common sequence polymorphism is found in the promoter of *ΔDNMT3B*, which affects promoter activity. In accordance with the notion that *ΔDNMT3Bs* is involved in early lung tumorigenesis, individuals carrying the T-type promoter, which has a higher promoter activity, had a >2-fold increased risk of lung cancer in a large case-control epidemiological study (22). Together, these data support the role of *ΔDNMT3Bs* in regulating promoter methylation during lung tumorigenesis.

Acknowledgements

This study was supported by the Department of Defense, Grant DAMD17-01-1-01689-1.

References

1. Jaenisch R: DNA methylation and imprinting: why bother? Trends Genet 13: 323-329, 1997.
2. Jones P and Gonzalgo M: Altered DNA methylation and genome instability: a new pathway to cancer? Proc Natl Acad Sci USA 94: 2103-2105, 1997.
3. Robertson KD and Wolffe AP: DNA methylation in health and disease. Nat Rev Genet 1: 11-19, 2000.
4. Surani MA: Imprinting and the initiation of gene silencing in the germline. Cell 93: 309-312, 1998.
5. Monk M, Boubelik M and Lehnert S: Temporal and regional changes in DNA methylation in the embryonic, extra-embryonic, and germ cell lineages during mouse embryo development. Development 99: 371-382, 1987.
6. Okano M, Xie S and Li E: Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19: 219-220, 1998.
7. Li E and Jaenisch R: DNA Alterations in Cancer. Ehrlich M (ed). Eaton Publishing, Natick, MA, pp351-365, 2000.
8. Okano M, Bell DW, Haber DA and Li E: DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. Cell 99: 247-257, 1999.
9. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA and Jones PA: The human DNA methyltransferases (DNMTs)1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 27: 2291-2298, 1999.
10. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H and Hrohoshi S: Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci USA 99: 10060-10065, 2002.
11. Oue N, Kuraoka K, Kuniyasu H, Yokozaki H, Wakikawa A, Matsusaki K and Yasui W: DNA methylation status of hMLH1, p16(INK4a), and CDH1 is not associated with mRNA expression levels of DNA methyltransferase and DNA demethylase in gastric carcinomas. Oncol Rep 8: 1085-1089, 2001.
12. Yakushiji T, Uzawa K, Shibahara T, Noma H and Tanzawa H: Over-expression of DNA methyltransferases and CDKN2A gene methylation status in squamous cell carcinoma of the oral cavity. Int J Oncol 22: 1201-1207, 2003.
13. Sato M, Horio Y, Sekido Y, Minna JD, Shimokata K and Hasegawa Y: The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines. Oncogene 21: 4822-4829, 2002.
14. Liu K, Wang YF, Cantemir C and Muller MT: Endogenous assays of DNA methyltransferases: evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells *in vivo*. Mol Cell Biol 23: 2709-2719, 2003.
15. Bachman KE, Park BH, Rhee I, Rajagopalan H, Herman JG, Baylin SB, Kinzler KW and Vogelstein B: Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell 3: 89-95, 2003.
16. Rhee I, Jair KW, Yen RW, Lengauer C, Herman JG, Kinzler KW, Vogelstein B, Baylin SB and Schuebel KE: CpG methylation is maintained in human cancer cells lacking DNMT1. Nature 404: 1003-1007, 2000.
17. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS and Huang TH: Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63: 6110-6115, 2003.
18. Jair KW, Bachman KE, Suzuki H, Ting AH, Rhee I, Yen RW, Baylin SB and Schuebel KE: *De novo* CpG island methylation in human cancer cells. Cancer Res 66: 682-692, 2006.
19. Girault I, Tozlu S, Lidereau R and Bieche I: Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 9: 4415-4422, 2003.
20. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB and Vogelstein B: DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416: 552-556, 2002.
21. Kim GD, Ni J, Kelesoglu N, Roberts RJ and Pradhan S: Co-operation and communication between the human maintenance and *de novo* DNA (cytosine-5) methyltransferases. EMBO J 21 4183-4195, 2002.
22. Shen H, Wang L, Spitz MR, Hong WK, Mao L and Wei Q: Identification of a new genetic variant in the novel *Dnmt3b* promoter and its association with promoter activity and risk of lung cancer. Cancer Res 62: 4992-4995, 2002.

APPENDIX B

Project 2.1 – IRB approved protocol

A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Solid Tumors Previously Treated with Standard Chemotherapy

PROTOCOL: WCI 1106-05

VERSION: August 21, 2006

Principal Investigator: John Kauh, M.D.

Co-Principal Investigator: Fadlo R. Khuri, M.D.

Study coordinator: Rivka Elbein, R.N.

Co-Investigators:

Medical Oncology:	**Michael Fanucchi, M.D.** **Dong Shin, M.D.** **Nabil Saba, M.D.**
Basic Science:	**Shi Yong Sun, Ph.D.**
Pathology:	**Anthony Gal, M.D.**
Radiology:	**William Small, M.D.**
Statistician:	**Andre Rogatko, Ph.D.**
Surgery:	**Amy Chen, M.D.**

Emory University
Winship Cancer Institute
1365C Clifton Road
Atlanta, GA 30322
404-778-5435

TABLE OF CONTENTS

INVESTIGATOR ROLES

Principle Investigator: John Kauh, MD
Assistant Professor of Hematology & Oncology
Winship Cancer Institutute
1365-C Clifton Road, NE
Atlanta, GA 30322
Phone: 404-778-2407
Email: john.kauh@emoryhealthcare.org

Co-Principle Investigator: Fadlo Khuri, MD
Professor of Hematology & Onocology
Winship Cancer Institutute
1365-C Clifton Road, NE
Atlanta, GA 30322
Phone: 404-778-4250
Email: fadlo.khuri@emoryhealthcare.org

Study Coordinator : Rivaka Elbein, RN
Research Nurse
Winship Cancer Institute
1365-C Clifton Road, NE
Atlanta, GA 30322
Phone : 404-778-5848
Email : rivka.elbein@emoryhealthcare.org

1. SYNOPSIS

1.1 TITLE:

A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Solid Tumors Previously Treated with Standard Chemotherapy

1.2 STUDY OBJECTIVES:

- Establish the maximum tolerated dose (MTD) of combination therapy with bexarotene and celecoxib.
    - The MTD is defined as the dose at which $\geq$ 33% of patients treated with bexarotene and celecoxib experience dose limiting toxicity (DLT).
- Measure expression of biomarkers in tumor samples take from subjects (before and after therapy with bexarotene and celecoxib) and relate to tumor response in an exploratory correlative analysis.
- Perform an exploratory analysis of antitumor activity of the combination of bexarotene and celecoxib in patients who develop a grade 3 or 4 hypertriglyeridemia within 4 weeks of initiation of therapy.

1.3 SUMMARY OF DESIGN:

This will be a Phase I dose escalation study

1.4 STUDY MEDICATION:

Commercially available bexarotene (Targretin) and celecoxib (Celebrex) will be prescribed to each patient who meets entry criteria and agrees to participate in this trial.

1.5 DOSE REGIMEN AND DURATION OF ADMINISTRATION:

Phase I Trial with dose escalation from 200 mg/m^2 Bexarotene daily and 200 mg BID of Celecoxib dose level I; 300 mg/m^2 Bexarotene and 200 mg BID of Celecoxib dose level II; 400 mg/m^2 Bexarotene and 200 mg BID of Celecoxib dose level III; 400 mg/m^2 Bexarotene and 400 mg BID of Celecoxib dose level IV. Treatment will continue until there is evidence of tumor progression.

1.6 NUMBER OF PATIENTS:

The study is expected to enroll approximately 36 patients. A maximum of 36 patients may be enrolled to this protocol.

1.7 SELECTION OF PATIENTS:

Patients with solid tumors who have received up to 3 lines of standard chemotherapy will be selected for enrollment in this trial.

1.7.1 Major Inclusion Criteria:

ECOG performance status 0-1

Tumor tissue accessible to serial biopsy

Patient willing to sign informed consent

Subject must be considered legally capable of providing his or her own Consent for participation in this study.

Biological plausibility of tumor targets to the combination

1.7.2 Major Exclusion Criteria:

ECOG performance status ≥ 2

Hemoglobin <8 gm/dL

Creatinine >2.0 mg/dL

Transaminases >3 times upper limit of normal

Total bilirubin >1.5 mg/dL

Triglycerides >200 mg/dL despite optimal medical management.

1.8 EFFICACY EVALUATIONS:

Modulation of biomarker expression in serial tumor samples obtained from subjects before and after therapy with bexarotene and celecoxib will be investigated in an exploratory correlative analysis. Immunohistochemical stains will be done at baseline, and after two cycles (4 week cycle) of treatment. Specifically the following candidate biomarkers will be examined:

- Up-regulation of PPAR-γ and RXR's (α,β,γ)
- Down-regulation of Cyclin D1, COX-2, and PGE-2

1.9 SAFETY EVALUATIONS:

Adverse event, physical examination, and clinical laboratory data will be collected throughout the study duration. Descriptive statistics for these safety parameters will be summarized.

2. BACKGROUND AND RATIONALE

Both bexarotene and celecoxib are currently being explored in combination with cytotoxic agents in several types of solid tumors. Bexarotene is an RXR specific retinoid that has been approved by the FDA for the treatment of cutaneous T-cell lymphoma (CTCL). It has been shown to be effective in phase I or phase II trials for the treatment of lung and breast cancer when used as a single agent or in combination with other chemotherapeutic agents, and is currently undergoing phase III trials in the United States and Europe. The intriguing properties of this compound include downregulation of cyclin D1, and upregulation of PPARγ in preclinical studies although its antitumor mechanisms are largely unclear. Data from two large randomized clinical trials (SPIRIT I & SPIRIT II) of bexarotene with or without chemotherapy in non-small call lung cancer (NSCLC) presented at the 2005 Annual American Society of Clinical Oncology meeting suggests benefit from the addition of bexarotene may be limited to patients who develop a

grade 3 or 4 hypertriglyeridemia despite aggressive use of cholesterol lowering agents prior to study entry.

Celecoxib, a selective COX-2 inhibitor, has been tested preclinically and found to inhibit COX -2 activity in various types of human cancer cells including head and neck, and lung cancer cells. It induces apoptosis of cancer cells in cell culture systems, inhibits tumor growth and carcinogenesis in animal models, and exhibits chemopreventive activity against colon cancer in patients. COX-2 expression is widely described in tobacco-related cancers and tumors of the GI tract, prostate, lung, and other solid tumors. Although the mechanisms by which celecoxib induces apoptosis and tumor regression remain unknown, we recently have revealed that celecoxib up regulates the expression of death receptor 4 (DR4) and 5 (DR5), which is involved in mediating celecoxib-induced apoptosis in human lung cancer cells, suggesting that DRs may serve as a novel target for celecoxib-mediated apoptosis and antitumor activity. Moreover, we have found that activation of PPARγ leads to DR up regulation, which may account for the mechanism underlying DR upregulation by celecoxib.

Since RXR forms a permissive heterodimer with PPARγ and bexarotene selectively activates RXR as well as increases PPARγ expression, it is plausible to speculate that the combination celecoxib and bexarotene will result in synergy on induction of DR expression and apoptosis in human cancer cells. Therefore, we propose a mechanistically and biologically driven study using celecoxib and bexarotene combination regimen for the treatment of solid tumors. The primary goals for this study are the establishment of a minimum effect of biological dose in these two relatively non-toxic agents, and the modulation in tumor samples of DR4, DR5, PPARγ, COX-2 expression and PGE2 level. Additionally, we will look at the effect of the combination on induction of apoptosis in solid tumor biopsy.

3. OBJECTIVES

1. Establish maximum tolerated dose (MTD) defined as the dose at which $\geq$ 33% of patients treated with bexarotene and celecoxib experience dose limiting toxicity (DLT).
2. Measure expression of biomarkers, with immunohistochemical methods, in tumor samples take from subjects (before and after therapy with bexarotene and celecoxib) and relate to tumor response in an exploratory correlative analysis. The specific candidate biomarkers include:

DR4	Cyclin D1
DR5	COX–2
PPARγ	PGE_2

3. Perform an exploratory analysis of antitumor activity of the combination of bexarotene and celecoxib in patients who develop a grade 3 or 4 hypertriglyeridemia within 4 weeks of initiation of therapy.

4. SUMMARY OF STUDY DESIGN:

This will be a Phase I trial with dose escalation. Dose levels will be established as follows:

- **Dose Level 1:** Bexarotene 200 mg/m^2 daily and celecoxib 200 mg BID
- **Dose Level 2:** Bexarotene 300 mg/m^2 daily and celecoxib 200 mg BID
- **Dose Level 3:** Bexarotene 400 mg/m^2 daily and celecoxib 200 mg BID
- **Dose Level 4:** Bexarotene 400 mg/m^2 daily and celecoxib 400 mg BID

A minimum of 3 and a maxium of 6 subjects will be enrolled in dose levels 1 and 2. A minimum of 6 and a maximum of 12 subjects will be enrolled in dose levels 3 and 4. The number of patients in the dose level 3 and 4 cohorts are increased as the higher dosages are felt offer an improved chance of meaningful anti-tumor activity. Safety will be assessed after all cohort members have received a treatment at the initial dose level.

Any nonhematologic grade 3 or 4 or hematologic grade 4 drug-related toxicities (NCI – Common Toxicity Criteria Version 3.0) occurring in the first 28 days of the study will be considered a dose limiting toxicity (DLT). Safety will be assessed after all subjects in each cohort have been treated for a minimum of 4 weeks and if the safety criteria have been met, enrollment in the next dose level may begin.

Dose escalation will be as follows for dose level 1 and 2:

- If none of the first 3 subjects experiences a DLT then the trial will proceed to enrollment at the next dose level.
- If one of the first 3 subjects experiences a DLT, then three additional subjects will be studied at the same dose level.
    - If one or more of these 3 new subjects experiences a DLT, then the MTD has been exceeded. And the study will not proceed to the next dose level.
    - If none of the these 3 new subjects experiences a DLT, then the study may proceed to the next dose level.
- If two or more of the first 3 subjects experiences a DLT, then the MTD has been exceeded and no further dose escalation will be performed.

Dose escalation will be as follows for dose level 3 and 4:

- If one or fewer the first 6 subjects experiences a DLT then the trial will proceed to enrollment at the next dose level.
- If two of the first 6 subjects experiences a DLT, then 6 additional subjects will be studied at the same dose level.
    - If two or more of these 6 new subjects experiences a DLT, then the MTD has been exceeded. And the study will not proceed to the next dose level.
    - If one or fewer of the these 6 new subjects experiences a DLT, then the study may proceed to the next dose level.

- If three or more of the first 6 subjects experiences a DLT, then the MTD has been exceeded and no further dose escalation will be performed.

Biopsies will be performed prior to treatment and repeated after 2 cycles (4 weeks per cycle) of treatment. Immuno-histochemical stains will be done at these intervals to access the biological markers.

Patients will receive a physical evaluation and routine laboratory safety tests at baseline, 2 weeks after the initiation of treatment, at 4 weeks and every 4 weeks thereafter while they remain in the trial.

We anticipate this study will be activated in October, 2005 and will be completed in April, 2007.

5. PATIENT SELECTION AND ELIGIBILITY

5.1 Inclusion Criteria

5.1.1 Inclusion Criteria for Definition of Study Population

5.1.1.1 Patients with solid tumors who have received up to 3 regimens of standard chemotherapy

5.1.1.2 Male or female over the age of 18

5.1.1.3 Willing to give informed consent

5.1.1.4 Willing to agree to have serial biopsies as called for in the protocol

5.1.1.5 Tumor accessible for serial biopsies

5.1.2. Inclusion Criteria for Safety Issues:

Patients must meet all of the following inclusion criteria:

5.1.2.1.Acceptable organ function defined as follows:

- Fasting serum triglyceride ≤200 mg/dL.
- Absence of hepatic dysfunction that is characterized by SGOT (AST) and SGPT (ALT) > 3 times upper limit of normal or serum bilirubin greater than the upper limit of normal.
- Hemoglobin ≥8 g/dL, WBC ≥2,000/mm^3, and platelets ≥50,000/mm^3.

5.1.2.2.Women of childbearing potential must have negative pregnancy test (serum β-HCG) with a sensitivity of at least 50 mIU/L within 48 hours prior to the initiation of treatment and must have used two reliable forms of effective contraception used simultaneously (strongly recommended that one of the two forms of contraception be non-hormonal) or must have been sexually abstinent for at least four (4) weeks prior to the negative pregnancy test through entry in the study.

5.1.2.3.Female patients and male patients with female partners of childbearing potential must agree to sexual abstinence or to practice two reliable forms of effective contraception used simultaneously (strongly recommended that one of the two forms of contraception be non-hormonal) during the entire period of Targretin capsule treatment and for at least one (1) month after treatment is discontinued.

5.1.2.4.Male patients with female sexual partners who are pregnant, possibly pregnant or who could become pregnant during the study must agree to use condoms during sexual intercourse during the entire period of Targretin capsule treatment and for at least one (1) month after the last dose of Targretin capsules.

5.2 Exclusion Criteria

5.2.1 Exclusion Criteria for Defining the Study Population

5.2.1.1 Age less than 18

5.2.1.2 Unwilling to undergo serial biopsies as required by the protocol

5.2.2. Exclusion Criteria for Safety Issues:

Patients meeting any one of the following exclusion criteria must be excluded:

5.2.2.1.Known hypersensitivity to bexarotene or other component of Targretin capsules.

5.2.2.2.Pregnancy, intent to become pregnant, or breast-feeding.

5.2.2.3.Risk factor for pancreatitis (e.g., prior pancreatitis, uncontrolled hyperlipidemia, excessive alcohol consumption, uncontrolled diabetes mellitus, biliary tract disease, and medications known to increase triglyceride levels or to be associated with pancreatic toxicity).

5.2.2.4.Systemic anticancer therapy of any kind within 14 days prior to initiating study medications.

5.2.2.5. Investigational therapy of any kind within 30 days prior to initiating study medications.

5.2.2.6. Systemic vitamin A in doses exceeding 15,000 IU/day within 14 days prior to initiating study medications.

5.2.2.7. Unwillingness or inability to minimize exposure to sunlight and artificial ultraviolet light while receiving Targretin capsules.

5.2.2.8. Known contraindication (according to product labeling) to the study drug(s) or protocol-required antilipid agent.

5.3. Prohibitions After Enrollment on Study

During the study, the following therapies are prohibited and may not be administered to patients being treated on this protocol:

5.3.1 Gemfibrozil (Lopid®), because of a documented drug-drug interaction that increased plasma levels of bexarotene and made the control of hypertriglyceridemia difficult.

5.4. Precautions After Enrollment on Study

During the study, the following therapies may be administered to patients but should be done so with caution:

5.4.1. Patients should avoid or be on stable doses of phenytoin, phenobarbital, rifampin, rifabutin, carbamazepine, dexamethasone, protease inhibitors, or other compounds that may affect the metabolism of bexarotene through an induction of drug-metabolizing enzymes.

5.4.2. Because co-administration may result in increased plasma concentrations of bexarotene, caution should be used when coadministering ketoconazole or other azole antifungals, erythromycin or other macrolide antibiotics, grapefruit juice, St. John's wort or other compounds that are known inhibitors of P450 3A4 drug metabolism.

5.4.3. Bexarotene may theoretically increase the rate of metabolism and reduce the plasma concentrations of compounds metabolized by P450 3A4, including oral contraceptives. Patients co-administered compounds metabolized by P450 3A4 should be monitored for evidence of subtherapeutic concentrations of the co-administered compounds.

5.4.4. Systemic use of other retinoid class drugs, beta-carotene compounds, or vitamin A doses more than 15,000 IU (5,000 mcg) per day (equivalent to approximately three times the Recommended Daily Allowance) for any indication.

5.4.5. Use of insulin, agents enhancing insulin secretion (e.g., sulfonylureas), and insulin-sensitizers (e.g., troglitazone). (Although hypoglycemia has not been associated with Targretin capsules, based on the mechanism of action Targretin capsules could enhance the action of these agents.)

5.4.6. Caution must be used if anticoagulants are given in conjunction with Tricor® to maintain prothrombin time at the desired level. See package insert.

6.0 TREATMENT PLAN

6.1 Overview of the Treatment Plan

Following baseline evaluation, patients who meet the inclusion and exclusion criteria set forth in this protocol may be enrolled in the trial. Patients will receive oral doses of the study medication based on the dose level to which they have been assigned. The dose level to which a patient is assigned is determined by the dose level cohort that is being tested when he or she enrolls in the trial. Celebrex will be self-administered twice daily and Targretin will be self-administered daily. Patients will be seen for follow-up visits following each 4 week treatment cycle. Treatment will continue until tumor progression is documented.

Patients will be seen and enrolled in the Winship Cancer Institute Medical Oncology clinics. Patients may be referred from outside referring physicians. Patients will be consented by the treating physician. Witnesses will be clinic nurses or other members present during the informed consent interview process. Patients may request information and decide to enroll at a later date.

6.2 Method of Administration

Both study drugs, Targretin and Celebrex, will be administered as oral capsules. Patients will self-administer the drugs on an outpatient basis. Compliance with therapy with be assessed by capsule counts at each follow-up visit. At the initial and each follow-up visit, subjects will be supplied with a sufficient quantity of study drugs for the next treatment cycle.

6.2.1. Method of Administration of Targretin Capsules

The study staff will compute the body surface area (BSA) from each patient's height and weight using a body surface area nomogram. The calculated absolute dose will be determined by multiplying the BSA in m^2 by the assigned mg/m^2 dose regimen. The amount of the total once-daily dose in mg to be administered should be computed to within the nearest 37.5 mg, using the appropriate number of 75 mg capsules. Details of dosing information (e.g., BSA, prescribed dose, date of dosing, any dose adjustments) are to be captured in the case report forms.

Targretin capsules, supplied as soft gelatin capsules, are to be taken orally with at least six (6) ounces of water or other fluid and should be taken with or immediately following the evening meal (a moderate or full meal). A nutritionally-defined liquid food such as Ensure® Plus may be substituted for a meal. If at all possible, other oral medications should not be taken within one hour before or one hour after ingesting Targretin capsules.

With regard to approval for use in the treatment of patients with CTCL, the U.S. package insert for Targretin capsules may be found in Appendix 2, current as of the date of this protocol version.

6.2.2 Method of Administration of Antilipid Agent

Treatment with a lipid-lowering agent is required from Day 1 of Targretin therapy. The choice of lipid lowering agent is left at the discretion of the investigator. This section provides information on the use of Lipitor as a lipid-lowering agent due to the large experience accumulated with Lipitor therapy and Targretin. Treatment with Lipitor must be initiated on or before Day 1. It is recommended that the patient be started on 40mg QD of Lipitor. For patients still requiring a greater

degree of lipid control, the dose of Lipitor may need to be increased as tolerated by the patient. It is recommended that the patient's lipid level be closely monitored by weekly blood chemistry assessment during the first four (4) weeks following the beginning of Targretin. See Section 6.4.3 for specific guidance on the monitoring and management of hypertriglyceridemia.

6.2.3 Method of Administration of Levothyroxin

If the patient is euthyroid at study entry, treatment with levothyroxin 0.05 mg must be initiated on Day 1. If the patient has been diagnosed with hypothyroidism before study entry and is on levothyroxin, the current dose of levothyroxin must be continued. Serum levels of T4 should be monitored on a monthly basis; the dose of levothyroxin should be adjusted to maintain the patient euthyroid.

6.2.4 Method of Administration of Celebrex

The dose of Celebrex to be administered will be determined by the patients dose-level cohort assignment. The U.S. Package Insert for Celebrex, current as of the date of this protocol version, is appended to this protocol (Appendix 3).

6.3 Treatment Regimen and Duration

Patients will receive doses of the study drugs based on their dose-level cohort assignment. The doses to be evaluated are:

- Dose Level 1: Bexarotene 200 mg/m^2 daily and celecoxib 200 mg BID
- Dose Level 2: Bexarotene 300 mg/m^2 daily and celecoxib 200 mg BID
- Dose Level 3: Bexarotene 400 mg/m^2 daily and celecoxib 200 mg BID
- Dose Level 4: Bexarotene 400 mg/m^2 daily and celecoxib 400 mg BID

Patients will continue to receive the study drugs until there is evidence of tumor progression.

Clinical Toxicity Scale

The clinical toxicity scale used in this study is the NCI Table of Common Terminology Criteria for Adverse Events v3.0 (CTCAE) found in Appendix 4. This toxicity scale will be used to determine when clinical toxicities, that are in the judgment of the Investigator attributable to the administration of either of the study drugs (i.e., Targretin or Celebrex capsules), necessitate a reduction in dose or a suspension or discontinuation of study drug.

A patient who experiences any visual disturbance should undergo an appropriate ophthalmologic evaluation, including a slit-lamp eye examination when appropriate. Any lens opacities occurring or worsening during the study should be followed until stabilized to the degree that they no longer need to be closely followed as judged by the Investigator, or until satisfactorily explained as not due to administration of study drug.

6.4.2 Study Drug Dose Adjustments

In the event of a Targretin capsule-related toxicity, the dose of Targretin capsules for an individual patient may be reduced from 400 to 300 mg/m^2/day, then to 200 mg/m^2/day, or suspended at any time, as necessary.

Any toxicity requiring a 2 week treatment interruption will result in removal of the patient from the study.

A patient who experiences a Grade 3 or 4 toxicity should be monitored at least weekly until the toxicity resolves or improves to NCI Grade 1 or 0 in the NCI Toxicity Table (except, for serum triglycerides, see Section 6.4.3); for ANC, to NCI Grade 2 or better [>1,000/mm3]; for hemoglobin, to NCI Grade 2 or better [>8 g/dL]; for platelets, to NCI Grade 2 or better [>50,000 mm3]; and for SGOT,

SGPT and serum bilirubin, to < 3 times upper limit of normal [in the range of NCI Grade 2, or better].

Dose adjustments of the other protocol-specified medications should be made according to product labeling in effect at that time and current standards of practice.

The Investigator must use the available data and exercise clinical judgment in deciding whether or not an adverse event is study drug-related (see Section 11.4 for guidelines for determining relatedness of adverse events). For a given toxicity, efforts should be made to identify which medication, if any, might be reasonably associated with and responsible for that toxicity. For Targretin capsules, the Investigator is referred to the Targretin Package Insert (PI) (see Appendix 2) and to the appropriate product labeling for the common adverse events known to be associated with this agent.

Some toxicities might be readily attributed to Targretin capsules (e.g., hypertriglyceridemia, headache, central hypothyroidism, asthenia, and mucocutaneous dryness). Patients should be encouraged to endure known, non-threatening toxicities and adverse events, when possible, without dose reduction. For example, mild to moderate bexarotene-associated symptoms such as headache and mucocutaneous dryness may be treated with supportive therapy (e.g., analgesics, nonsteroidal anti-inflammatory agents, skin and eye lubricants and moisturizers, etc.). Bexarotene-induced hyperlipemia may be managed according to the guidelines provided in Section 6.4.3. Central hypothyroidism may be managed with exogenous thyroid hormone supplementation (see Section 6.4.4). Bexarotene-induced toxicities are generally reversible after drug discontinuation and tolerance may develop to some retinoid side effects over time. However, if a patient cannot tolerate the assigned Targretin capsule dose, the dose may be adjusted by the Investigator according to the guidelines above in this section.

The decision regarding treatment adjustment or discontinuation from study drug therapy for suspected drug-associated toxicities, made in the patient's best interest, should follow the protocol guidelines but ultimately rests with the Investigator.

Targretin capsules should be discontinued at the first clinical evidence of any toxicity that is considered to be related to Targretin capsules administration and reasonably considered to be serious and/or life-threatening as judged by the Investigator. Under these circumstances, such patients should not be restarted on Targretin capsules unless there is some indication of patient benefit. In these cases, the reason for re-instituting Targretin capsules must be clearly indicated in the case report form.

6.4.3. Monitoring and Management of Hypertriglyceridemia

Targretin capsules have been associated with altered blood lipid profiles, especially increased serum triglycerides. Significant elevation of serum triglyceride levels is associated with an increased risk for developing acute pancreatitis, especially in the presence of other risk factors for pancreatitis (e.g., prior history of pancreatitis, uncontrolled hyperlipidemia, excessive alcohol consumption, uncontrolled diabetes mellitus, biliary tract disease, and medications known to increase triglyceride levels or to be associated with pancreatic toxicity).

In the Phase II-III studies, about 70% of patients with cutaneous t-cell lymphoma (CTCL) who received Targretin capsules at an initial dose of ≥300 mg/m^2/day had fasting triglyceride levels greater than 2.5 times the upper limit of normal. About 55% had values over 800 mg/dL. Cholesterol elevations above 300 mg/dL occurred in approximately 60% and 75% of patients with CTCL who received an initial dose of 300 mg/m^2/day or >300 mg/m^2/day, respectively.

Decreases in HDL cholesterol to less than 25 mg/dL were seen in about 55% and 90% of patients at these initial dose levels, respectively. The effects of triglycerides, HDL cholesterol, and total cholesterol were reversible with cessation of therapy, and could generally be mitigated by dose reduction or concomitant antilipid therapy.

Acute pancreatitis, in association with serum triglyceride levels exceeding 770 mg/dL, has been reported in about 2% of patients receiving Targretin capsules; however, these patients were not on aggressive treatment with Lipitor and levothyroxine. If fasting triglycerides are elevated during Targretin capsule treatment, antilipid therapy should be adjusted and, if necessary, the dose of Targretin capsules should be reduced or suspended.

Fasting triglycerides must be within the age-adjusted normal limit or normalized with appropriate intervention prior to initiating Targretin capsule therapy. As detailed in Section 6.2.2, treatment with atorvastatin (Lipitor) must be initiated on or before Day 1. **Gemfibrozil (Lopid®) should not be used in conjunction with Targretin capsules because of a documented drug-drug interaction that increased plasma levels of bexarotene and made the control of hypertriglyceridemia difficult.**

In general, it is recommended that fasting lipid determinations should be performed post-baseline no less frequently than at weekly intervals until the lipid response to Targretin capsules is established, which usually occurs within two to four weeks, and then at four-week intervals thereafter. For this reason, this protocol specifies that post-baseline fasting lipid determinations should be performed weekly until Week 4, and then every four (4) weeks thereafter throughout the time of the follow-up visit that is to occur approximately four weeks after discontinuation of Targretin capsules. Attempts should be made to maintain serum triglyceride levels below 800 mg/dL.

Atorvastatin (Lipitor) has been used effectively to control bexarotene-induced hypertriglyceridemia. In Phase II-III clinical trials of Targretin capsules in patients with refractory or persistent CTCL, atorvastatin was used by 48% (73/152) of patients. In an interim analysis of data from a Phase II clinical trial of Targretin capsules in patients with metastatic breast cancer, fenofibrate was the recommended antilipid agent and antilipid therapy was initiated for 67% (52/90) patients.

If the patient has triglyceride levels at screening that are not within the age-adjusted norm, the patient should begin on Lipitor 40 mg qd and monitored until the triglyceride levels have normalized. Once the triglyceride levels have been normalized, the patient may be entered into the study.

After the first week of Targretin treatment, the triglycerides should be measured. If the triglyceride level is 400-800 mg/dL, the Lipitor dose should be increased to 80 mg/d. If the patient is receiving a Targretin dose of > 300 mg/m^2 and the triglyceride level is 800-1200 mg/dL, the Lipitor dose should be increased to 80 mg daily and a Targretin dose reduction to 300 mg/m^2 should be considered. If the triglyceride level is greater than 1200 mg/dL, the Targretin should be held for one week and restarted at a dose of 300 mg/m^2. The triglyceride levels should be measured at Weeks 2, 3, and 4. If the triglycerides are not controlled, modify the Lipitor and Targretin doses as described above. If the LFT's are greater than three times the upper limit of normal, the Lipitor should be discontinued and the Targretin dose should be reduced.

Patients should be regularly reminded to avoid risk factors that can lead to pancreatitis, such as heavy alcohol consumption and/or dehydration. Additionally, patients should be cautioned to be alert for any symptoms suggestive of the onset of pancreatitis, such as abdominal pain, nausea and vomiting.

Table .1 Targretin Dosage Adjustments		
Targretin Dose (mg/dL)	Triglyceride Level (mg/dL)	Action
200	<400	None
	400 – 800	Increase Lipitor to 80mg
	800 – 1200	€ Increase Lipitor to 80mg € Decrease Targretin to 150mg/m^2
300	<400	None
	400 – 800	Increase Lipitor to 80mg
	800 – 1200	€ Increase Lipitor to 80mg € Decrease Targretin to 200mg/m^2
400	<400	None
	400 – 800	Increase Lipitor to 80mg
	800 – 1200	€ Increase Lipitor to 80mg € Decrease Targretin to 300mg/m^2

6.4.4 Monitoring and Management of Hypothyroidism

Targretin capsules have been associated with central hypothyroidism. In the Phase II-III studies in patients with CTCL, Targretin capsules induced biochemical evidence of clinical hypothyroidism in about half of all patients treated, causing a reversible reduction in thyroid hormone (total thyroxine [total T4]) and thyroid stimulating hormone (TSH) levels. The incidences of decreases in TSH and total T4 were about 60% and 45%, respectively, in patients treated at the recommended initial dose of 300 mg/m^2/day. Biochemical hypothyroidism was not always associated with clinical symptoms and/or might have been associated with symptoms that were not readily attributed to hypothyroidism but may have been attributed to the underlying disease. The incidence of hypothyroidism reported as an adverse event in these studies was 29%. A total of 37% of patients received treatment with thyroid hormone replacement. The

central hypothyroidism was reversible, with recovery to normal levels of TSH occurring as early as seven days after discontinuation of Targretin capsules.

In this current study, TSH and total T4 levels are obtained at baseline (Day 1); the T4 level is repeated every four weeks until discontinuation of Targretin capsules. Since failure to treat hypothyroidism may exacerbate the side effect of hypertriglyceridemia and render control of elevated triglyceride levels more difficult, all patients must be on levothyrixine (either 0.05 mg/day or their current replacement dose) at the time of study entry; the dose of levothyroxin should be modified based on T4 levels obtained during the course of the study. This dose should be adequate because the dose of thyroid hormone required to treat patients with bexarotene-induced hypothyroidism in other clinical trials did not differ substantially from the dose required to treat hypothyroidism from other causes. Because the hypothyroidism observed in association with bexarotene is central in nature, the dose required to render the patient euthyroid should be guided by the normalization of the total T4 level and cannot be guided by the suppression of TSH.

6.4.5 Gastrointestinal Protective Agents

All patients started on Celebrex shall receive a gastrointestinal protective agent(H_2 blocker or proton pump inhibitor) for any gastric distress.

6.5 Treatment Termination

The Investigator must guard the patient's welfare and should discontinue study drug treatment at any time that this action appears to be in the patient's best interest. A patient is free to withdraw from study treatment and participation at any time for any reason. The specific reasons and circumstances for all treatment terminations must be documented in the case report forms.

Criteria for terminating Targretin capsule therapy include, but are not limited to, the following:

- Clinical toxicity (see definition in Section 6.3.1) attributable to Targretin capsules that does not resolve or improve to NCI Grade 1 or 0 within two (2) weeks of stopping the study medication responsible for the toxicity.

- Pregnancy.

- SGOT (AST), SGPT (ALT), or serum bilirubin attributable to Targretin capsules exceeding three times the upper limit of normal should prompt consideration of stopping Targretin capsules, but the overall risk-benefit ratio for the individual patient should be taken into consideration.

- General or specific changes in the patient's condition that render the patient unacceptable for further treatment with Targretin capsules, in the judgment of the Investigator.

- The patient or patient's physician is free to discontinue Targretin capsule treatment and take the patient off study at any time, if this is believed to be in the patient's best interest.

Additional criteria for discontinuing Celebrex include, but are not limited to:

- Hypersensitivity reaction

- Gastric bleeding

7. STUDY PROCEDURES

Study Procedure Schedule Overview

	Pre-Study	Day 1	Weekly	Week 4	Week 8	Every 4 Weeks	Every 8 Weeks
CBC	X						
Chemistries	X						
Lipid Profile	X		X Until Stable	X	X	X	
TSH, Free T4	X			X	X	X	
Pregnancy Test if applicable	X						
Imaging	X						X
Tumor Biopsy	X			X	X		
Lipitor		X Continue throughout study participation					
Levothyroxin		X Continue throughout study participation					

7.1 Study Subject Recruitment

Treating physicians will approach patients who the physician feels is a potential candidate for study entry. If the patient wishes to pursue study entry, the patient will then meet with the study coordinator who will review in depth the study requirements. The patient will also be given a copy of the informed consent to take home, so that the patient may take as much time as they need to make an informed decision.

7.2 Medical History and Physical Examination

At the initial visit each subject will have a complete medical history taken and physical examination performed. The physican examination may be performed by a licensed physician, nurse practitioner ("NP") or physician's assistant ("PA") under the direction and supervision of the investigator, co-investigator or sub-

investigator. Both normal and abnormal findings should be noted. Vital signs, including pules and respiration rate, temperature and blood pressure as well as the patient's weight should be recorded at each visit.

7.3 Laboratory Evaluations

At baseline and at each follow-up visit, patients shall have a standard laboratory safety battery performed, including serum chemistries, CBC and differential, and urinalysis. A list of the specific individual tests to be performed and the normal values for each test for the Emory Clinic laboratory are provided in Appendix 5.

7.4 Imaging Test Evaluations

Within 4 weeks of starting Targretin and celecoxib and following every two cycles (8 weeks), patients shall undergo either MRI or CT Scan imaging, as appropriate for the location and type of tumor being evaluated. Bi-dimensional tumor measurements shall be made and recorded. Objective response will be measured by RECIST criteria.

7.5 Biopsy

Each patient will undergo a tumor biopsy at baseline and following two cycles (week 8). The biopsy technique will be appropriate to the size and location of the tumor being studied. Biopsy specimens will be sent for immunohistochemical analysis (IHC).

IHC analysis will be a primary technique in this study since it can be used to examine several biomarkers in RXR and COX-2-mediated signaling pathway using paraffin embedded tissue samples. IHC will be performed with appropriate controls according to a standard procedure used in our previous studies [1, 2].

All of the antibodies are commercially available in Santa Cruz Biotech Inc (Santa Cruz, CA), Research Diagnostics Inc (Flanders NJ), Cell Signaling Technology (Beverly, MA), and Biomeda Corp (Foster City, CA).

To evaluate the expression level of each marker by immunohistochemistry, we will quantify the expression of these proteins using digitized images by Image Analysis System as we have described previously [3, 4]. We have developed the system so that the microscopic stage can be computer driven and digitized images can be stored on discs with their coordinates in memory. We will examine the whole tissue slides and look for the expressed area. The relative signal intensity (RSI) of stained area with each antibody will be scored from three best representatives in high-power field (x 400) and the average RSI of the staining will be calculated in each samples [2].

Core needle biopsies of tumor samples will be taken using sterile technique and will be performed by physicians certified to perform biopsies. For lesions other than superficial lesions, all biopsies will be performed with the assistance of radiology guidance, either ultrasound or CT. Prior to biopsy all patients will have partial thromboplastin times, prothrombin times , and platelet counts measured to minimize risk of bleeding. All biopsies will also be performed under sterile technique and will also be performed by physicians familiar in with the biopsy techniques.

7.6 Pregnancy Testing

Every female patient of childbearing potential shall have a pregnancy test performed at baseline and all follow-up evaluations. A negative pregnancy test is required within 48 hours of beginning study treatment.

7.7 Withdrawal of Patient from the Study

Patients will be removed from the clinical trial for the following reasons:

1. Documented progression of malignant disease either clinically or by radiological studies.
2. The treating physician feels that discontinuation of the trial is in the best interest of the patient's health.
3. The patient wishes to withdraw consent from participation in the clinical trial.

Once a patient is removed from the study, no further contact with the patient will be initiated by the study personnel unless issues regarding patient safety arise.

8. STUDY SCHEDULE

A matrix schedule showing all visits and required testing and study procedures is shown in Appendix 5.

8.1 Pre-Study (Screening Visit)

During this visit the investigtor and the research nurse shall evaluate the patient's eligibility for entry into the trial. Eligible patients shall have the required initial biopsy and imaging studies scheduled. Informed consent to participate in the trial will be obtained.

8.2 Baseline (Day 1) Visit

The required medical history and physical examination will be prepared, blood will be drawn for the required laboratory safety test will be obtained. A pregnancy test will be performed and the results obtained prior to dispensing any study drug. The initial supply of Targretin will be determined by calculating the patient's body surface area and determining the number of capsules that will provide the

daily dose that most closely matches the dose level to which the patient was assigned. Prescriptions for Celebrex, Lipitor and any other required drug are to be given to the patient. The patient should also be scheduled for the first follow-up visit, which will occur following the initial 4-week treatment cycle.

8.3 Study Visits During Treatment

Patients will be scheduled for a study visit at the conclusion of every 4-week treatment cycle. At each study visit the patient will be examined, questioned regarding the occurrence of any adverse effects, have blood drawn for laboratory safety tests and be scheduled for MRI or CT imaging. At the end of the second 4-week treatment cycle a biopsy will be performed.

8.4 Follow-up Visit

A final follow-up visit will be scheduled 4 weeks following the discontinuation of the last study drug. This visit will be scheduled for all patients regardless of the reason for discontinuation of study drugs (e.g tumor progression, adverse event, etc.) At this visit the patient will be examined and questioned regarding the occurrence of any adverse events since the discontinuation of study drug. Laboratory safety tests will also be performed at this visit.

8.5 Long-Term Follow-up Evaluations

No evaluations will be performed after the follow-up visit, unless required to document the course of an adverse event.

9.0 STUDY DRUG INFORMATION

9.1 Investigational New Drug (IND) Application

The use of bexarotene in this study will be justified by cross referencing Ligand Pharmaceuticals' IND of bexarotene.

9.2 Targretin Capsule Drug Information

With regard to approval for the treatment of patients with CTCL, the U.S. Package insert for Targretin capsules may be found in Appendix 2.

9.2.1 Targretin Capsule Formulation

Targretin capsules for oral administration will be supplied as follows:

75 mg capsules: Off-white, oblong, soft gelatin capsules in bottles and imprinted with "Targretin."

Active Ingredient: Bexarotene

Chemical Name: 4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl) ethenyl]-benzoic acid

Molecular Weight of Bexarotene: 348.48

Other Names for Drug Substance: LGD1069, LG100069

Excipients: Each capsule contains polyethylene glycol 400 NF, polysorbate 20 NF, povidone USP, and butylated hydroxyanisole NF. The capsule shell contains gelatin NF, sorbitol special-glycerin blend, and titanium dioxide USP.

Solubility: Bexarotene is insoluble in water and has limited solubility in vegetable oils and ethanol.

9.2.2 Targretin Capsule Dispensing and Accountability

At each visit, the appropriate number of Targretin capsules sufficient for continuation of treatment until the next scheduled visit will be dispensed to the patient. In general, the quantity dispensed should not exceed one month of treatment except as necessary because of the indivisible packaging consisting of 100 capsules per bottle. The capsules must be dispensed in Ligand's containers to assure stability of the drug.

At each visit at which Targretin capsules are dispensed and at the conclusion of the study, patients must be instructed to return all unused capsules in partially or completely full bottles in order to adequately assess each patient's compliance with dosing instructions. Capsule counts should be compared to the amount of study drug the patient should have taken in the preceding study interval. Empty medication bottles must also be returned for accounting. When the patient returns capsules or bottles, the date of return will be recorded on the Drug Inventory Form. Every effort should be made to obtain return of all unused capsules and dispensed bottles. If such effort fails, a dated note explaining the reason for the failure to collect the drug should be made on the Drug Inventory Form.

Returned bottles should not be redispensed to the patient at any time. At each visit at which Targretin capsules are dispensed, a new supply of study drug should be provided to the patient.

At the conclusion of the study, all capsules and bottles will be inventoried and returned to Ligand or its designee by a traceable shipment method. Any missing capsules must be explained on the Cumulative Drug Inventory Form. The total number of capsules used or returned plus those explained as lost should equal the number of capsules shipped to the site. Shipping receipts from receiving or sending drug bottles should be retained by the investigational site as part of the study records.

9.2.3 Targretin Capsule Storage

Investigational drug supplies must be stored in a secure, locked area. Patients should be instructed to store their bottles of Targretin capsules with the caps tightly closed in a safe area at 2° to 25° C (36° to 77° F). Targretin capsules should not be stored near heating devices, high temperatures or humidity, or where children or pets have access to them. Targretin capsules should be protected from sunlight.

9.2 Antilipid Drug Information

Atorvastatin (Lipitor) antilipid agent (see Section 6.2.2) is to be procured commercially under prescription by the Investigator.

9.3 Levothyroxin Drug Information

Levothyroxin is to be procured commercially under prescription by the investigator.

9.4 Celebrex Drug Information

Celebrex will be procured commercially under prescription by the investigator.

10. DATA ANALYSIS AND STATISTICAL INTERPRETATION

All data will be tablulated. Association of changes in expression of biomarkers in tumor samples before and after therapy with tumor response will be tested using Fisher exact tests and Mann-Whitnet tests depending on whether the expression of the biomarker is continous or ordinal. Ordinary logistic regression will be used

to study this association after adjusting for the dose level received by the patients.

11. ADVERSE EVENTS

The treating physician should follow all patients with adverse events, regardless of severity, until resolution is satisfactory.

11.1 Adverse Events Definitions

Adverse Event (AE):
An adverse event is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product, which may or may not have a causal relationship with this treatment.

Serious Adverse Event (SAE):
Serious adverse event is any adverse event occurring at any dose that results in any of the following outcomes: death, a life-threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Life-threatening is any adverse event that places the patient or subject, in the view of the investigator, at immediate risk of death from the reaction as it

occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

Unexpected/Unlabeled Adverse Event:

An unexpected or unlabeled event is an adverse event, the nature or severity of which is not consistent with the applicable product information (e.g., package insert for an approved indication or Investigator's Brochure for an unapproved indication).

11.2 Reporting of Adverse Events

All serious adverse events (defined in section 11.1), whether or not deemed study-related or expected, must be reported by the Principal Investigator or designee to the HSRRB and/or USAMRMC Human Research Protection Office within 24 hours (one working day) by telephone. A written report must follow as soon as possible, which includes a full description of the event and any sequelae. This includes serious adverse events that occur any time after the inclusion of the subject in the study (defined as the time when the subject signs the informed consent) up to 30 days after the subject completed or discontinued the study. The subject is considered completed either after the completion of the last visit or contact (e.g., phone contact with the Investigator or designee). Discontinuation is the date a subject and/or Investigator determines that the subject can no longer comply with the requirements for any further study visits or evaluations (e.g., the subject is prematurely discontinued from the study). An adverse event temporarily related to participation in the study should be documented whether or not considered to be related to the test article. This definition includes intercurrent illnesses and injuries and exacerbations of preexisting conditions. Include the following in all IND safety reports: Subject identification number and initials; associate investigator's name; subject's date of birth, gender, and ethnicity; test article and dates of administration; signs/symptoms and severity; date of onset; date of resolution or death;

relationship to the study drug; action taken; concomitant medication(s) including dose, route, and duration of treatment, and date of last dose.

All serious adverse events (AEs) occurring with any patient participating in this clinical trial must be reported to John Kauh, M.D. or **Fadlo Khuri, M.D.** as described below. The site will supply as much information (study number, patient initials, patient study number, onset date, relationship, patient demographics, event, dosing regimen of the study drug) as is available at the time of the initial telephone call or Fax. Reports of all serious adverse events must be communicated to the appropriate Institutional Review Board (IRB) or ethical review committee and/or reported in accordance with local laws and regulations.

FOR ANY UNEXPECTED/UNLABELED SERIOUS ADVERSE EVENT, CONTACT [John Kauh, M.D. or Fadlo Khuri, M.D.], IMMEDIATELY [WITHIN 24 HOURS].

John Kauh, M.D.
Asst. Professor of Hematology and Oncology
Winship Cancer Institute
1365C Clifton Road
Atlanta, GA 30322
Office Phone: 404 778 2407
Office Fax: 404 778 4389

Fadlo Khuri, MD
Co-Principal Investigator
Winship Cancer Institute
1365C Clifton Road
Atlanta, GA 30322
Office Phone: 404 778 4486
Office Fax: 404 778 4389

Follow-up information to serious, adverse events that are unexpected or unlabeled must be provided to **John Kauh, M.D. or Fadlo Khuri, M.D.** promptly as it becomes known.

For any fatal, life-threatening and/or serious adverse event, whether or not associated with the use of the drug, the approving Institutional Review Board (IRB) must be notified on a timely basis. A written report of all serious adverse events and deaths will be submitted by the Investigator to the IRB. In addition, the IRB must be appraised of *all* adverse events by written report on a periodic and timely basis, at least annually. In this report, the Investigator will advise whether or not the adverse event is judged to be related to the study drug administration. At the time this report is submitted to the IRB, a copy should be provided to Ligand Pharmaceuticals Inc.

Unanticipated problems involving risk to subjects or others, serious adverse events related to participation in the study and all subject deaths should be promptly reported by phone (301-619-2165), by email (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the U.S. Army Medical Research and Materiel Command, Human Subjects Research Review Board (HSRRB). A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21702-5012

In addition, the Principal Investigator is responsible for submitting all adverse event reports to the HSRRB and/or USAMRMC, Deputy for Compliance and Quality (301-619-2165) on an ongoing basis.

The death of any subject during the study or within 30 days of study completion, regardless of the cause, must be reported within 24 hours by telephone to the HSRRB and/or USAMRMC, followed by a full written report as soon as possible. If an autopsy is performed, the report must be provided to the Sponsor.

As described in 21 CFR 312.32, physicians holding an IND are responsible for reporting (and those who have received an exemption are requested to report) to FDA within 15 calendar days, any adverse events associated with the use of a drug that are both serious and unexpected/unlabeled. Any unexpected fatal or life-threatening events associated with the use of a drug should be reported to

FDA by telephone or Fax as soon as possible but no later than 7 calendar days after the Investigator's initial receipt of the information.

In addition, for any serious adverse event associated with the use of a drug that is unexpected/unlabeled, Ligand Pharmaceuticals Inc. must be notified by the Principal Investigator, or designee, by Fax to Ligand Medical Safety at (858) 550-1860 at the time that FDA is notified. These reports may be filed utilizing the Form FDA 3500 (MedWatch Form). In the instance of a medical emergency, notify Ligand Pharmaceuticals Medical Safety by telephone at 1-800-577-3017 or 1-858-550-7750.

For comparator drugs/secondary suspects (concomitant medications), all unexpected/unlabeled, serious adverse events should be forwarded by the Investigator to the appropriate product manufacturer.

11.3 Classification of Adverse Events by Severity

The Investigator must categorize the severity of each adverse event according to the following guidelines:

Mild:

Grade I NCI Common Toxicity; or if not found in the Common Toxicity tables, an adverse event that is asymptomatic or barely noticeable to the patient; not interfering with patient's daily activity performance or functioning; generally not requiring alteration or cessation of study drug administration; and/or ordinarily not needing therapeutic intervention.

Moderate:

Grade II NCI Common Toxicity; or if not found in the Common Toxicity tables, an adverse event of sufficient severity as to possibly make the patient moderately uncomfortable; possibly influencing the patient's daily activity performance or functioning; generally not impairing the patient's ability to continue in the study; and/or possibly needing therapeutic intervention.

Moderately Severe:

Grade III NCI Common Toxicity; or if not found in the Common Toxicity tables, an adverse event generally causing severe discomfort; significantly influencing the patient's daily activity performance or functioning; generally requiring alteration or cessation of study drug administration; and/or generally requiring therapeutic intervention.

Severe:

Grade IV NCI Common Toxicity; or if not found in the Common Toxicity tables, an adverse event that is considered to be life-threatening; resulting in significant disability or incapacity; and/or representing the worst possible occurrence of that event.

11.4 Classification of Adverse Events by Relationship to Study Drug Administration

The relationship of each adverse event to the study drug administration will be assessed by the Investigator after careful consideration, and according to the following guidelines:

NO, NOT RELATED

This category is applicable to those adverse events which are clearly due to extraneous causes (concurrent drugs, environment, etc.) and do not meet the criteria for drug relationship listed under PROBABLY NOT; POSSIBLY; PROBABLY; AND YES, RELATED.

PROBABLY NOT RELATED

This category applies to those adverse events which are judged to be unlikely to be related to the study drug administration. An adverse event may be considered to be PROBABLY NOT RELATED when it meets at least two (2) of the following criteria:

a) It does not follow a reasonable temporal sequence from administration of the study drug.
b) It could readily have been produced by the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.
c) It does not follow a known or expected response pattern to the study drug.
d) It does not reappear or worsen when the study drug is re-administered.

POSSIBLY RELATED

This category applies to those adverse events which are judged to be perhaps related to the study drug administration. An adverse event may be considered POSSIBLY RELATED when it meets at least one (1) of the following criteria:

a) It follows a reasonable temporal sequence from administration of the study drug.
b) It could not readily have been produced by the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.
c) It follows a known or expected response pattern to the study drug.

PROBABLY RELATED

This category applies to those adverse events which are felt with a high degree of certainty to be related to the study drug administration. An adverse event may be considered PROBABLY RELATED if it meets at least two (2) of the following criteria:

a) It follows a reasonable temporal sequence from administration of the study drug.
b) It could not be reasonably explained by the known characteristics of the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.
c) It disappears or decreases on cessation or reduction in study drug dose. There are exceptions when an adverse event does not disappear upon discontinuation of the drug, yet drug relatedness clearly exists (e.g., bone marrow depression, fixed drug eruptions, tardive dyskinesia, etc.).
d) It follows a known or expected response pattern to the study drug.

YES, RELATED

This category applies to those adverse events which are incontrovertibly related to study drug administration. An adverse event may be assigned to this category if it meets at least the first three (3) of the following criteria:

a) It follows a reasonable temporal sequence from administration of the study drug.

b) It could not be reasonably explained by the known characteristics of the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.

c) It disappears or decreases on cessation or reduction in study drug dose. There are exceptions when an adverse event does not disappear upon discontinuation of the drug, yet drug relatedness clearly exists (e.g., bone marrow depression, fixed drug eruptions, tardive dyskinesia, etc.).

d) It follows a known or expected response pattern to the study drug.

e) It reappears or worsens when the study drug is re-administered.

12. ADMINISTRATIVE ASPECTS

12.1. Institutional Review Board

This protocol and the proposed informed consent form must be reviewed and approved by the appropriate Institutional Review Board (IRB), and by the HSRRB and/or USAMRMC, Human Research Protection Office, prior to the start

of the study. A copy of the IRB approval letter of the protocol, any amendments, and the informed consent form should be supplied to the Sponsor-Investigator prior to starting the study. During the course of the study, the Sub-Investigator or Sponsor-Investigator shall make timely and accurate reports to the IRB on the progress of the trial, at intervals not exceeding one year, as well as satisfying any other local IRB regulations regarding reporting. Copies of all reports to and correspondence with and from the IRB must be provided to the Sponsor-Investigator.

It is the Sub-Investigator's obligation to maintain an IRB correspondence file, and to make this available for review by the Sponsor-Investigator or his/her representatives.

12.2. Informed Consent

The proposed informed consent form must be in compliance with regulatory regulations. The proposed informed consent form must contain a full explanation of the purpose and nature of the study, a description of the procedures, the possible advantages, risks, alternate treatment options, and a statement of confidentiality of patient study records, a statement regarding voluntary compensation and availability of treatment in the case of injury, an explanation of whom to contact about the research, the patient's rights, and notification that participation is voluntary and refusal will involve no penalty or loss of medical benefits. These requirements are in accordance with the Federal Regulations as detailed in the 21 CFR 50.25 and the Declaration of Helsinki. It should also indicate by signature that the patient, or where appropriate, legal guardian/representative, permits access to relevant medical records by the Sponsor-Investigator, by representatives of Ligand Pharmaceuticals Inc. and by representatives of the U.S. Food and Drug Administration (FDA), U.S. Department of Defense, or other applicable regulatory agency.

Sub-Investigators or the Sponsor-Investigator will be responsible for obtaining written informed consent from potential patients prior to any study specific screening and entry into the study. A copy of the signed document will be provided to the patient, and a copy will be maintained with the patient's Case Reporting Forms (CRFs), or in the study documentation notebook. The original will be retained by the Investigator along with the CRFs.

Patients will be consented by the treating physician. Witnesses will be clinic nurses or other members present during the informed consent interview process. Patients may request information and decide to enroll at a later date.

12.2.1 Withdrawal of Informed Consent

As described in the informed consent form, participating patients in the trial may withdraw consent at anytime. The patients are asked to discuss their reasoning with the study coordinator or the principle investigator prior to withdrawing consent, however the patient may opt not to speak to the coordinator or the principle investigator. Once a patient withdraws from the study, we will ask the patient to be followed for at least 4 weeks to determine any adverse events. If a patient withdraws consent and they wish their tumor biopsy samples be destroyed they simply need to make a request to the study coordinator or the principle investigator and the patient's wishes will be carried through.

12.3. Laboratory Accreditation

Any laboratory facility to be used for analysis of routine clinical laboratory samples required by this protocol must provide evidence of adequate licensure or accreditation. Licensure/accreditation and reference values and/or normal ranges for the test results must be provided to the Sponsor-Investigator.

12.4. Data Recording and Case Report Forms (CRFs)

Case Report Forms will be developed by the Sponsor-Investigator for the collection of all study data. One copy is to be retained in any Sub-Investigator's files. The original copy must be sent to (or collected by) the Sponsor-Investigator. The forms should be firmly printed or written legibly, using typewriter or black ball-point pen. They must be completed in a timely manner in order for a patient to continue under this protocol.

It is the obligation of any Sub-Investigator to review each page of the CRF and to sign the designated and appropriate forms as the study's authority. CRF completion may be formally delegated to other study personnel. However, the Sponsor-Investigator must be informed in writing of the name of such persons and the scope of their authority.

12.5 Drug Accountability and Prescription

The study drugs (Targretin and Celebrex) are to be prescribed only by the Principal Investigator or physician sub-investigators. Under no circumstances will the Investigator(s) allow the investigational drug to be used other than as directed by this protocol.

The Investigator must maintain accurate records accounting for the receipt of Targretin supplies and for the disposition of the drug. Documentation of the disposition of Targretin should consist of a dispensing record or Drug Inventory form, including the identification of the person to whom the drug is dispensed, the quantity and the date of dispensing, and any unused drug returned. This record is in addition to any drug accountability information recorded on the case report forms. At the termination of the study or at the request of Ligand Pharmaceuticals Inc., the Investigator must return any unused Targretin and all partially dispensed or empty containers to Ligand or its designee. This return will be documented at Ligand. Ligand or its designee must be notified in writing at

least 30 days prior to the intended date of disposal of any study records related to this protocol.

All other study drugs (other than Targretin) will be prescribed by the Principal Investigator or physician Sub-Investigators named on the Form FDA 1572.

12.6. Record Retention

The Investigator must keep on file protocols, amendments, IRB approvals, all copies of Form FDA 1572, all correspondence, and any other documents pertaining to the conduct of the study for a minimum of two (2) years after notification by USAMRMC, Human Research Protection Office of either FDA approval or discontinuation of the IND. The Sponsor-Investigator should be notified in writing at least 30 days prior to the disposal of any study records related to this protocol. The investigator and other appropriate study staff will be responsible for maintaining all documentation relevant to the study. Such documentation includes:

- Case Report Forms—must be legible, accurate, and up-to-date.
- Copies of all Serious AE reporting forms faxed to the USAMRMC, Human Research Protection Office.
- Participant Files—should substantiate the data entered in the CRFs with regard to laboratory data, participant histories, treatment regimens, etc.
- Participant Exclusion Log—should record the reason any participant was screened for the study and found to be ineligible.
- Drug Dispensing Log—should record the total amount of study drug received and returned to sponsor, and the amount distributed and returned or destroyed. This information must agree with the information entered in the CRFs.
- Informed Consent Forms—completed consent forms from each participant must be available and verified for proper documentation.
- Informed Consent Log—must identify all participants who signed an Informed Consent Form so that the participants can be identified by audit.

Data recorded on Case Report Forms (CRFs) must be legible and complete. CRFs will be completed on a timely basis. The individual making the correction on the CRF must initial and date the correction. The investigator must review all

final and corrected CRFs. Corrected copies of CRFs will be filed with the corresponding original.

If a subject's medical record is needed, it will be requested by the Principal Investigator or his designee. The requesting investigator (i.e., either the PI or his designee) will assume responsibility for medical record abstraction, which will be performed by an oncology research nurse and the PI. The PI is a medical oncologist.

12.7 Subject Identification

A unique master subject ID will be assigned to each individual participating the study. A password protected secured file will be created to store the cross reference list between the master ID and confidential patient information such as name, birth date, and social security number (if available), etc. Master ID will be used throughout the trial and in database for patient identification purpose. Confidential patient information will be used only when it is necessary such as in patient care setting.

12.8 Disclosure of Data

All information obtained as a result of this study or during the conduct of this study will be regarded as confidential. Disclosures (i.e., any release of information to any third party not noted herein) of any, not previously known to be public, information and/or results of the investigation for publication or by oral or poster presentation shall not be made earlier than 30 days after submission of the proposed material to Ligand Pharmaceuticals Inc. for inspection, unless Ligand consents to earlier disclosure. The Investigator will take appropriate cognizance of Ligand's suggestions before disclosure for publication or presentation, consistent with protection of Ligand's right to its confidential data.

12.9 Publication Agreement:

The Investigator expressly agrees that no interim publication of data will occur without the review of Ligand Pharmaceuticals. Data will be reviewed by the collaborating biostatistician prior to publication. HSRRB and/or USAMRMC, Human Research Protection Office will have 30 days to review all definitive publications, such as manuscripts and book chapters, and a minimum of 10-15 days to review all abstracts.

12.10 Gender and Minority Inclusion

Women and minorities will be actively recruited to participate in the trial. We expect that the ethnic distribution of the enrolled participants will reflect the local ethnic mixture of the surrounding community.

12.11 Ethical and Legal Considerations

This study will undergo full approval in accordance with the human surveillance requirements of the WCI IRB and the HSRRB. Blood samples will be obtained for the evaluations as described in the protocol. Tissue samples obtained at the time of two scheduled biopsies. Measures will be taken to ensure confidentiality of participant information. Tissue samples will be collected prospectively during the trial. Data collected on paper forms will be stored in locked file cabinets with restricted access. Data collected on electronic media will be stored in computer files with restricted password access. All staff members in the study will be informed prior to employment and at regular intervals of the necessity for keeping all data confidential. Computers will not be accessible to the public and will be located in locked offices. Subjects will be assigned a separate study number to protect subject identification. No patient identifiers will be used in any publications of this research. Data will be maintained indefinitely. When the time comes to dispose of the data, all database files will be deleted.

13 DATA SAFETY MONITORING PLAN

As required by the Winship Cancer Institute (WCI), Data Safety Monitoring Plan (DSMP), a protocol-specific DSMP has been prepared and is presented below:

13.1 Protocol Title

A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Solid Tumors Previously Treated with Standard Chemotherapy

13.2 Principal Investigator:

John Kauh, MD
Assistant Professor of Hematology and Oncology
Winship Cancer Institute – Emory University School of Medicine
1365C Clifton Road, Bldg. C
Atlanta, GA 30322
404-778-2407

Co-Principal Investigator:

Fadlo Khuri, MD
Professor and Associate Director for Translational and Clinical Research
Chief Medical Officer
Winship Cancer Institute – Emory University School of Medicine
1365C Clifton Road
Atlanta, GA 30322
404-778-5435

The Principal Investigator and Co-Principal Investigator have passed the Emory IRB Certification Examination as required by the WCI DSMP.

13.3 General Provisions

This study will be conducted in compliance with the Winship Cancer Institute Data Safety Monitoring Plan. The definitions, responsibilities and procedures specified there-in are incorporated by reference into this protocol-specific DSMP.

13.4 Responsible Individual

The Principal Investigator, John Kauh, MD, will be the individual responsible for assuring implementation of this DSMP.

13.5. Study Approval and Review of Endpoints

This protocol has been approved by the appropriate WCI Working Group and is subject to review and approval by the WCI Clinical and Translational Research Committee, including annual progress reviews.

13.6 Study Monitoring

The investigator is responsible for the continuous monitoring of all participants in the trial.

13.6.1 Medical Monitor

The medical monitor is defined as a qualified physician, other than the principal investigator, not associated with the protocol, able to provide medical care for research volunteers for conditions that may arise during the conduct of the study, and who will monitor the volunteers during the conduct of the study. The medical monitor will review all serious and unexpected adverse events associated with this protocol and provide an unbiased written report of the event within ten (10) calendar days of the initial report. At a minimum, the medical monitor will comment on the outcomes of the adverse event (AE) and relationship of the AE to the study. The medical monitor should also indicate whether he/she concurs with the details of the report provided by the study investigator. Reports for events determined by either the investigator or medical monitor to be possibly or definitely related to participation and reports of events resulting in death should be promptly forwarded to the HSRRB.

The medical monitor for this study will be Dr. Jean Khoury, M.D.
Emory University
1365 Clifton Road, NE
Atlanta, GA 30322
Phone: 404-7784250 or 404-778-2407
FAX: 404-778-5520 OR 404-778-5676
email: hkhoury@emory.edu

The medical monitor will forward reports to the U.S. Army Research and Material Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland, 21702-5012.

13.6.2 External Monitoring

External monitoring of the trial will be the responsibility of the WCI Monitoring Office. The trial will be monitored according to the following schedule:

- Initial subject ;
- When enrollment reaches two subjects or 20% of the projected accrual yearly, whichever is greater;
- Two additional subjects or 10% of accrual after a successful first year, whichever is greater.
- At the conclusion of the study.
- Additional monitoring may be performed in the event of Serious Adverse Events (see DSMP 2.4.1 for the definition of an SAE) if deemed necessary by the monitoring committee or if requested by the Principal Investigator.)

Monitoring reports will be prepared according the WCI standard procedures and will be reviewed by the Monitoring Committee as required by the WCI DSMP.

13.7 Serious Adverse Event Reporting

SAEs must be reported as required by the WCI DSMP (Section 2.4). In addition, since some activities will be carried out at the General Clinical Research Center (GCRC), all adverse events will be promptly entered into the GCRC database according to the established procedure.

13. 8. Data Safety Monitoring Board

Based on the criteria established in the WCI DSMP, a separate Data Safety Monitoring Board is not required for this trial.

13.9 Privacy

This trial will be conducted in accordance with the Emory University IRB policy regarding HIPAA compliance.

Measures will be taken to ensure confidentiality of participant information. Data collected on paper forms will be stored in locked file cabinets with restricted access. Data collected on electronic media will be stored in computer files with restricted password access. All staff members in the study will be informed prior to employment and at regular intervals of the necessity for keeping all data confidential. Computers will not be accessible to the public and will be located in locked offices. Subjects will be assigned a separate study number to protect subject identification. No patient identifiers will be used in any publications of this research. Data will be maintained indefinitely. When the time comes to dispose of the data, all database files will be deleted.

14 STUDY ADMINISTRATION AND INVESTIGATOR OBLIGATIONS

14.1 Roles and Responsibilities of Key Study Personnel

14.1.1 Principle Investigators:

The principle and co-principle investigators are charged with reviewing and ensuring all study materials are up to date and accurate. They are required to evaluate and review all adverse events and severe adverse events. The principle investigators will also be available to answer all study related questions.

14.1.2 Co-Investigators:

The co-investigators will be responsible for providing study related analytical and patient care services within their respective areas of expertise.

14.1.3 Medical Onocologists:

Will over see the direct patient care issues of study participants and evaluate study subjects for toxicity and response to the study agents.

14.1.4 Study Coordinator:

Will be charged with screening, consenting, and enrolling patients into the study. The coordinator will also be responsible for ensuring all adverse events and severe adverse events are documented and reported in a timely fashion.

14.1.5 Basic Scientists:

Will perform the correlative studies outline in this protocol.

14.1.6 Pathologist:

Will review all study related pathological samples to ensure uniform evaluation of all study samples.

14.1.7 Radiologist:

Will review all study related radiology studies to ensure uniform evaluation of studies. The radiologist will also assist with image guided tumor biopsies required by the study.

14.1.8 Bio-Statistician:

Will review and analyze study data as outlined in section 10 (Data Analysis and Statistical Interpretation) of this study protocol.

14.1.9 Surgeon:

Will assist with study required biopsies requiring a surgeon.

14.1 Study Amendments

Any amendments cannot be enacted unless approved by the HSRRB and/or USAMRMC, Human Research Protection Office. All revisions made to protocols previously approved by the IRB will be submitted to the IRB for approval prior to implementation of the revision. If the IRB decides to disapprove a research activity, it shall include in its written notification a statement of the reasons for its decision and give the investigator an opportunity to respond in person or in writing. No changes to the protocol will be initiated unless also approved by the Human Subjects Research Review Board and/or USAMRMC, Human Research Protection Office.

14.2 Protocol Deviations

In the event of a protocol deviation, the principal investigator will notify the Department of Defense, MD Anderson, the study's Medical Monitors (Drs. Khoury and Waller) within 24 hours of discovery.

14.3 Termination of Study

The HSRRB and/or USAMRMC, Human Research Protection Office and Emory University Winship Cancer Center will retain the right to terminate the study and remove all study materials from the study site at any time. Specific instances that may precipitate such termination are as follows:

- Unsatisfactory participant enrollment with regard to quality or quantity
- Deviation from protocol requirements, without prior approval from HSRRB or Emory University
- Inaccurate and/or incomplete data recording on a recurrent basis
- The incidence and/or severity of adverse drug events in this or other Studies indicating a potential health hazard caused by the treatment

14.4 Subject Records

HSRRB and/or USAMRMC, Human Research Protection Office, Emory University or its representatives may have access to subject records.

14.5 Risks/Benefits

Participants may or may not receive medical benefit from the treatment on this study.

The risks of participating in this study include:

1. Side effects from Targretin – Refer to section 6.4.2
2. Side effects of celecoxib – Increased risk of severe cardiovascular complications, particularily at doses ≥ 400 mg BID. Other side effects of celecoxib include: nausea, diarrhea, indigestion, headache, bleeding in the stomach, allergic reaction, ulcers in the mouth, stomach pain, liver damage (including liver failure), and/ or decreased kidney function (including kidney failure).
3. Risks of biopsy – Pain, bleeding, and/or infection.

Participants will not be financially responsible for any study-related tests outside of accepted standard of care follow-up.

References:

1. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991; 324:1-8.
2. Shin DM, Charuruks N, Lippman SM, et al. p53 protein accumulation and genomic instability in head and neck multistep tumorigenesis. Cancer Epidemiol Biomarkers Prev 2001; 10:603-9.
3. Hoffmann TK, Nakano K, Elder EM, et al. Generation of T cells specific for the wild-type sequence p53(264-272) peptide in cancer patients: implications for immunoselection of epitope loss variants. J Immunol 2000; 165:5938-44.
4. Chikamatsu K, Albers A, Stanson J, et al. P53(110-124)-specific human CD4+ T-helper cells enhance in vitro generation and antitumor function of tumor-reactive CD8+ T cells. Cancer Res 2003; 63:3675-81.

APPENDIX C

Project 2.1 – IRB approval letter

EMORY UNIVERSITY | Institutional Review Board

John Kauh MD
SOM: Winship Cancer Institute
Hematology & Oncology
1365 Clifton Rd., NE, Bldg. C, Rm C3012
Atlanta, GA 30322

RE: **NOTIFICATION OF RENEWAL APPROVAL**
PI: John Kauh MD
IRB ID: **046-2006**
TITLE: WCI1106-05 A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Tumors Previously Treated with Standard Chemotherapy

DATE: January 05, 2007

Renewal Review Type: Expedited

The continuing approval request referenced above was reviewed and APPROVED by the IRB. This approval is valid from **12/28/2006 until 12/27/2007**. Thereafter, continued approval is contingent upon the submission of a renewal form that must be reviewed and approved by the IRB prior to the expiration date of this study.

Any serious adverse events or issues resulting from this study should be reported immediately to the IRB and to any sponsoring agency (if any). Amendments to protocols and/or revisions to informed consent forms/process must have approval of the IRB before implemented.

All inquires and correspondence concerning this protocol must include the IRB number and the name of the Principal Investigator.

If you have any questions or concerns, please contact the IRB office at 404-712-0720 or at email address irb@emory.edu. Our web address is http://www.emory.edu/IRB. Thank you.

Sincerely,

Ann Haight, MD
Vice Chair, Institutional Review Board

CC: Rhoda Campbell; Leah Holloway

Emory University
1256 Briarcliff Road, 3rd Floor, North Wing
Atlanta, Georgia 30306
Tel 404.712.0720
Fax 404.727.1358
IRB@emory.edu

This approval is valid from 12/28/2006 until 12/27/2007.

PAGE 2 of RENEWAL APPROVAL

IRB ID: **046-2006** DATE: January 05, 2007

TITLE: WCI1106-05 A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Tumors Previously Treated with Standard Chemotherapy

The above referenced protocol renewal was approved including the information below. Please review this information for

Informed Consents Associated with this protocol:

Version Date	Description
8 /21/2006	Main
8 /21/2006	HIPAA

Personnel		**Human Subjects Education Certification Information**
Gal, Anthony	Co-Investigator	CITI - MED Refresher (19-Nov-2006)
Rogatko, Andre	Co-Investigator	CITI- MED 01, 02, 03, 07, 12, 14, 17 (07-March-2006)
Graves, Ellen	Other	CITI - MED 1, 2, 3, 7, 12, 14,17 VA GCP3, 5, 6, 7, 8 (11-May-2006) SHB 1, 2, 3, 4, 5, 6, 17 (11-May-2006)
Sun, Shi-Yong	Co-Investigator	CITI- MED Refresher (07-March-2006)
Bryant-Fletcher, Toshiwa	Other	CITI - MED Refresher (05-June-2006)
Saba, Nabil	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14 (24-Oct-2003) - SHB 1, 2, 3, 4, 5, 6, 7, 17 (27-Jul-2006)
Small, William C.	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (10-Jun-2005)
Chen, Amy	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (02-June-2006) SHB 1, 2, 3, 4, 5, 6, (02-June-2006)
Khuri, Fadlo	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (11-Aug-2004) Refresher (10-Sept-2006)
Fanucchi, Michael P.	Co-Investigator	CITI - MED Refresher (27-Jul-2006)
Elbein, Rivka	Research Coordinator	CITI - MED 1, 2, 3, 7, 11, 12, 14, 17 (10-Jul-2006) SHB 1, 2, (10-Jul-2006)
Kauh, John	Main Investigator	CITI - MED 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 (07-Oct-2003) Refresher (7-OCT-2005)

Shin, Dong	Co-Investigator	CITI MED Refresher (19- May-2006)
Clark, Jane B.	Other	CITI - MED Refresher 1, 2, 3, 7, 12, 14, 17 (08-Jun-2005)
Holloway, Leah	Other	CITI - MED 1, 2, 3, 7, 12, 13, 14, 17 (21-Feb-2006) VA GCP 3, 5, 6, 7, 8 (10-Oct-2006)
Warner, David B.	Research Coordinator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (18-Jul-2006)
Hamilton, Austin	Research Coordinator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (07-Aug-2006)
John-Reid, Laurell	Study Nurse	CITI - MED 1, 2, 3, 7, 12, 14, 17 (04-Aug-2006)
Campbell, Rhoda	Protocol Contact	CITI - MED 1, 2, 3, 7, 12 14, 17 (16-Aug-2006) GCP 3, 5, 6, 7, 8, (16-Aug-2006)
Harper, Shequila	Research Coordinator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (09-Oct-2006)

Number of Approved Emory Subjects 36 **(This number indicates the number of subjects you can consent.)**

Sites

Crawford Long Hospital
Winship Cancer Center

Radiation

ApproveDt	RadAuthorization
2 /8 /2006	RMH 761

EMORY
WINSHIP CANCER INSTITUTE

INFORMED CONSENT FOR CLINICAL RESEARCH

STUDY TITLE: WCI1106-05 A PHASE I BIOLOGIC STUDY OF BEXAROTENE (TARGRETIN®) AND CELECOXIB IN PATIENTS WITH SOLID TUMORS PREVIOUSLY TREATED WITH STANDARD CHEMOTHERAPY

PARTICIPATING INSTITUTIONS:

- Winship Cancer Institute - Emory University Hospital
- Crawford Long Hospital

STUDY SUPPORTERS:

- This protocol is partially funded by a research grant from the U.S. Department of Defense

PRINCIPAL INVESTIGATOR:

- John Kauh, MD

CO-PRINCIPAL INVESTIGATOR:

- Fadlo R. Khuri, MD

INTRODUCTION:

This is a clinical trial, a type of research study. Your study doctor will explain the clinical trial to you. Clinical trials include only people who choose to take part. Please take your time to make your decision about taking part. You may discuss your decision with your friends and family. You can also discuss it with your health care team. If you have any questions, you can ask your study doctor for more explanation.

You are being asked to take part in this study because you have a solid tumor that has been treated previously with chemotherapy.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to test the safety of the combination of bexarotene and celecoxib and see what effects (good or bad) it may have on your tumor, to determine the smallest dose that may lead to a shrinkage of your tumor, and to determine the highest dose of the combination of bexarotene and celecoxib that can be given without causing severe side effects.

This research is being done because we do not know how these two drugs, given in combination, may affect solid tumors.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

Approximately 36 people will take part in this study.

WHAT WILL HAPPEN IF I TAKE PART IN THIS RESEARCH STUDY?

When you enroll into the trial you will be placed in a group of 3 to 12 patients. All the patients in a particular group will be treated with the same doses of bexarotene and celecoxib. The doses of bexarotene and celecoxib that you get will depend on when you enter the study. The dose will be increased in groups of 3-12 patients until the maximum tolerated dose is found. This means that you may get a higher dose than the patients treated before you or a lower dose than patients treated after you. You will be assigned a dose when you enter the study. Once a group of patients has been treated at a certain dose level, all patients will be monitored for a minimum of 4 weeks to evaluate for safety. Once a dose level has been determined to be safe, the next group of patients will be treated with the next dose level. Toxicity due to therapy will be assessed by your physician through:

- History and examinations
- Blood tests

All the drugs come in pill form and you will take Bexarotene once a day and celecoxib twice a day. The pills should be taken about the same time of day each day.

- Dose Level 1: Bexarotene 200 mg/m^2 (dose determined by body size) daily and celecoxib 200 mg. twice a day
- Dose Level 2: Bexarotene 300 mg/m^2 daily and celecoxib 200 mg. twice a day

- Dose Level 3: Bexarotene 400 mg/m^2 daily and celecoxib 200 mg. twice a day
- Dose Level 4: Bexarotene 400 mg/m^2 daily and celecoxib 400 mg. twice a day

STUDY PROCEDURES

Study Procedure Schedule Overview

	Pre-Study	Day 1	Weekly	Week 4	Week 8	Every 4 Weeks	Every 8 Weeks
CBC	X						
Chemistries	X						
Lipid Profile	X		X Until Stable	X	X	X	
TSH, Free T4	X			X	X	X	
Pregnancy Test if applicable	X						
Imaging	X						X
Tumor Biopsy	X			X	X		
Lipitor		X Continue throughout study participation					
Levothyroxin		X Continue throughout study participation					

Before beginning treatment, a biopsy (a small piece of your cancer will be removed with either a needle or minor surgery) will be done. After 8 weeks of therapy with celecoxib and bexarotene another biopsy will be done. We are taking small pieces of your cancer to perform special tests on the cancer cells to see if celecoxib and bexarotene are doing anything to fight your cancer at a molecular level.

Before you start taking the chemotherapy drugs, you will also receive another three prescriptions drugs to take:

- Pepcid or Zantac – to protect your stomach from possible side effects of celecoxib.
- Thyroxine – you will need to supplement your thyroid hormone levels as bexarotene can cause low thyroid levels as a side effect.

- Lipitor – you will need to take Lipitor to prevent an increase in your cholesterol levels as bexarotene is known to increase cholesterol levels as a side effect.

The above drugs will not be provided by this study. You will get a prescription to obtain these drugs at your local pharmacy. The drugs will need to be paid for by you or your health insurance.

If you take part in this study, you will have the following tests and procedures:

- Procedures that are part of regular cancer care and may be done even if you do not join the study;
  - History and Physical Examination
  - Routine lab tests of blood
  - Imaging (either CT or MRI Scan) to measure your tumor

- Standard procedures being done because you are in this study:
  - Removal of a small piece of your tumor before starting on the study and after 8 weeks of treatment with bexarotene and celecoxib.

HOW LONG WILL I BE IN THE STUDY?

You will be in the study for a minimum of 8 weeks or as long as a year, provided you wish to continue participating in the trial and your cancer does not get worse.

The researcher may decide to take you off this if you experience a serious side effect, if the investigator determines it is in your best interest, or if new information becomes available that suggests it would be in your best interest.

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher and your regular doctor first.

CAN I STOP BEING IN THE STUDY?

Yes. You can decide to stop at any time. Tell the study doctor if you are thinking about stopping or decide to stop. He or she will tell you how to stop safely. It is important to tell the study doctor if you are thinking about stopping so any risks from stopping the treatment can be evaluated by your doctor. Another reason to tell your doctor that you are thinking about stopping is to discuss what follow up care and testing could be most helpful for you.

The study doctor may stop you from taking part in this study at any time if he/she believes it is in your best interest; if you do not follow the study rules; or if the study is stopped.

WHAT SIDE EFFECTS OR RISKS CAN I EXPECT FROM BEING IN THE STUDY?

You may have side effects while on the study. Everyone taking part in the study will be watched carefully for any side effects. However, there may be other side effects that we cannot predict. Side effects may be mild or very serious. Your health care team may give you medicines to help lessen side effects. Many side effects go away soon after you stop taking the drugs. In some cases, side effects can be serious, long lasting, or may never go away.

You should talk to your study doctor about any side effects that you have while taking part in the study.

Celecoxib (Celebrex®)

Celecoxib (Celebrex®) is approved by the U.S. Food and Drug Administration for use as an anti-inflammatory and analgesic for relief of the signs and symptoms of rheumatoid and osteo-arthritis in adults. It is not approved for the indication for which it is being tested.

Risks and side effects include:

Very Common (in more than 5% of patients)

- Diarrhea
- Upset stomach
- Headache
- Symptoms similar to a cold

Common (in 1% to 5% of patients)

- Abdominal pain
- Nausea
- Back pain
- Swelling of the arms and legs
- Dizziness
- Rash

Uncommon but serious side effects (in less than 1%)

- Severe allergic reactions
- Bleeding of the stomach

In addition, although celecoxib has not been directly implicated in increasing a persons risk for strokes and heart attacks, related drugs like Vioxx have been shown to increase a person's risk. As a result the U.S. Food and Drug Administration no longer permit the sale of Vioxx in the United States.

Targretin®

Targretin® is approved by the U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphoma. It is not approved for the indication for which it is being tested.

Risks and side effects include:

Very Common

- Reversible decrease in thyroid activity (approximately 50%)
- Elevation of cholesterol (doses less than or equal to 300 mg/m^2 per day – approximately 25%; greater than 300 mg/m^2 per day - 45%)
- Elevation of triglycerides (doses less than or equal to 300 mg/m^2 per day – approximately 28%; greater than 300 mg/m^2 per day - 45%)

Very Common (in more than 5% of patients)

- Headache
- Muscle weakness
- Infection
- Abdominal pain
- Chills
- Fever
- Back pain
- Rash
- Dry skin
- Hair loss
- Low blood counts
- Nausea
- Vomiting
- Loss of appetite
- Swelling of the extremities
- Insomnia
- Damage to liver

Common (in 1% to 5% of patients)

- Pneumonia
- Severe headache
- Infection

- Peeling of the skin
-

Uncommon but serious side effects (in less than 1%)

- Inflammation of the pancreas (pancreatitis)

Lipitor is a medication commonly used to lower cholesterol levels in patients with high cholesterol. High cholesterol levels over a long period of time can increase a person's risk of having a stroke or heart attack. Millions of people have safely taken Lipitor without significant problems but there are potential rare but serious side effects:

1. Liver damage can occur in up to 2% of patients receiving Lipitor. This damage is reversible and the liver will get better after you stop taking Lipitor. You will be closely monitored with blood tests while receiving Lipitor and the Lipitor will be discontinued if a serious abnormality in your liver blood tests occur.
2. Muscle damage has been rarely reported in less than 1% of patients receiving Lipitor. When Lipitor is stopped the muscle damage will improve over time without treatment. In about 5% of patients who take Lipitor, patients develop muscle aches but no permanent muscle damage is done and the muscle pain resolves after Lipitor is stopped.

Synthroid, also known as Levothyroxine, is used to replace or supplement thyroid hormone in people who have a slow thyroid or are missing their thyroid gland due to surgery. Synthroid is very well tolerated in the vast majority of people taking it and millions of people have taken it without significant problems. When you are taking Synthroid, you will be closely monitored with blood tests to make sure you are receiving the correct dose. Side effects associated with Synthroid use are usually due to too much Synthroid. These symptoms can be:

- Fatigue
- Increased appetite
- Weight loss
- Heat intolerance
- Headache
- Nervousness
- Irritability
- Insominia (trouble falling asleep)
- Tremors
- Increased heart rate and blood pressure
- Diarrhea
- Liver test abnormalities
- Flushing
- Menstrual irregularities
- Impaired fertility

You may experience some or none of the above side effects with Synthroid. To minimize the chances of you experiencing side effects, you will receive blood tests to closely monitor Synthroid levels so that our dosage can be adjusted properly. In addition to the above potential side effects, in very rare cases people have had an allergic reaction to Synthroid. There is no way to predict if you will have an allergic reaction but you will be closely monitored and if signs of an allergic reaction develop you will stop taking Synthroid immediately.

Risks of Procedures:

Tumor biopsy: You will be asked to undergo two biopsies as part of this study. A needle will be inserted into your tumor and some tissue will be removed. The physician will apply local anesthesia there and then perform the biopsy. You will be admitted to the hospital as an outpatient to have the biopsy. The risks of biopsy include pain, bleeding, and potential allergic reaction to the numbing medication used before the biopsy. There is also a small risk of infection. You be required to sign a separate consent form before a biopsy is done.

Blood samples: Blood withdrawal may cause pain and bruising at the site of vein puncture, inflammation of the vein, and infection; care will be taken to avoid these complications.

Risks of MRI

MRI uses magnetic fields and radio waves to "see" internal organs and soft tissue without the use of x-rays. Both safe and painless, MRI has no known side effects. MRI create detailed images of areas of your body.

A small proportion of people develop short-lived reactions during the dye administration, including nausea, headache, hot flashes, and heart palpitations. These symptoms usually resolve on their own within minutes. A smaller group of people are actually allergic to the dye and may develop a rash, hives, difficulty breathing, and in extreme cases, the patient may stop breathing, the heart may stop, and the patient may die. You will be closely monitored during the procedure, and if any allergic reaction develops, you will be treated immediately.

Risk of CT scan

CT scans are painless procedures that are safe for most patients. During the scan, you will lay flat with your body in a long metal cylinder that people who fear closed spaces sometimes find frightening. Patients can be observed at all times by the operators and can be assisted if necessary. You will be moved out of the machine if requested.

Patients at risk for injury from MRI or CT scans are those with pacemakers, aneurysm clips (metal clips on the wall of a large artery), or shrapnel

fragments. Welders and metal workers are also at risk for injury because of possible metallic foreign bodies in the eye. The MRI and CT machine makes a thumping sound while operating.

Risk of Radiation

You will receive a dose of radiation while you are undergoing scans. Radiation exposure is an unavoidable part of the procedure, but the level of exposure is low. These procedures would normally be performed as part of your medical care, and the number of scans required for patients on the study is no greater than for those having conventional treatment. The radiation resulting from scans is similar to the amount of natural radiation from living at a moderately high altitude for six months.

Reproductive risks: Both men and women will be included in this study. Because the drugs in this study might affect an unborn baby, you should not become pregnant or father a baby while in this study. Pregnant women must not take part in this study; neither should women who plan to become pregnant during the study. There may be risks which are currently unforeseen or unknown. All patients must use an effective mechanical or barrier birth control method while in this study. Examples of effective birth control are: a condom or a diaphragm with spermicidal jelly, oral, injectable, or implanted birth control, or abstinence. It is important you understand that you need to use birth control while on this study. Woman who can become pregnant must have a pregnancy test before taking part in this study.

If you are a woman capable of becoming pregnant (not surgically sterile or post-menopausal), you must have a negative pregnancy test before beginning treatment. In addition, you must not be nursing an infant during this study.

If you or your partner becomes pregnant while in this study you must tell the study doctor immediately. The doctor will advise you of the possible risks to your unborn child and discuss options for managing your pregnancy with you. Because of possible risks to your unborn child, the study drug will be stopped permanently

For more information about risks and side effects, ask your study doctor.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

Taking part in this study may or may not make your health better. While doctors hope this treatment will be more useful against cancer compared to the usual treatment, there is no proof of this yet. We do know that the information from this study will help patients with solid tumors in the future.

WHAT OTHER CHOICES DO I HAVE IF I DO NOT TAKE PART IN THIS STUDY?

Your other choices may include:

- Getting treatment or care for your cancer without being in a study
- Taking part in another study
- Chemotherapy alone

Talk to your doctor about your choices before you decide if you will take part in this study.

WILL MY MEDICAL INFORMATION BE KEPT PRIVATE?

We will do our best to make sure that the personal information in your medical record will be kept private. However, we cannot guarantee total privacy. Your personal information may be given out if required by law. If information from this study is published or presented at scientific meetings, your name and other personal information will not be used.

People other than those doing the study may look at your medical chart and study records. Agencies that make rules and policy about how research is done have the right to review these records. So do agencies that pay for the study. Those with the right to look at your study records include staff members involved with carrying out this study, the U.S. Food and Drug Administration, the U.S. Department of Defense, the Emory University IRB, representatives of the companies who provide the drugs used in this study (Ligand Pharmaceuticals and Pfizer Pharmaceuticals). Records can also be opened by court order. We will keep your records private to the extent allowed by law. We will do this even if outside review occurs. We will use a study number rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.

WHAT ARE THE COSTS OF TAKING PART IN THIS STUDY?

You and/or your health plan/ insurance company will need to pay for some or all of the costs of treating your cancer in this study. Some health plans will not pay these costs for people taking part in studies. Check with your health plan or insurance company to find out what they will pay for. Taking part in this study may or may not cost your insurance company more than the cost of getting

regular cancer treatment. The research staff will do their best to make you aware of what costs are not to be billed to you and instruct you to contact them if billing errors occur.
Targretin will be provided free of charge by Ligand Pharmaceuticals. The cost of Celebrex will be covered by study funding. The biopsies are paid for by a department of defense grant.

You will not be paid for taking part in this study.

For more information on clinical trials and insurance coverage, you can visit the National Cancer Institute's Web site at http://cancer.gov/clinicaltrials/understanding/insurance-coverage . You can print a copy of the "Clinical Trials and Insurance Coverage" information from this Web site.

Another way to get the information is to call 1-800-4-CANCER (1-800-422-6237) and ask them to send you a free copy.

WHAT HAPPENS IF I AM INJURED BECAUSE I TOOK PART IN THIS STUDY?

It is important that you tell your study doctors, Dr. John Kauh or Dr. Fadlo Khuri, if you feel that you have been injured because of taking part in this study. You can tell the doctor in person or call him/her at 404-778-4250.

If you are hurt or become sick as a result of your participation in this study, you can receive medical care at an Army hospital or clinic free-or-charge. You will be treated only for injuries that are caused directly by the research study. The Army will not pay for your transportation to and from the hospital or clinic. If you have questions about this medical care, talk with Dr. John Kauh or Dr. Fadlo Khuri. If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact Dr. John Kauh or Dr. Fadlo Khuri. If the issue cannot be resolved contact the U.S. Army Medical Research and Material Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663 or 301-619-2221.

You or your insurance company will be charged for continuing medical care and /or hospitalization.

We will give you emergency care if you are injured by this research. However, Emory University has not set aside funds to pay for this care or to compensate you if you are injured. If you believe you have been injured by this research, you should contact Dr. John Kauh or Dr. Fadlo Khuri at (404) 778-4250.

In the case of injury resulting from this study, you do not lose any of your legal rights to seek payment by signing this form.

WHAT ARE MY RIGHTS IF I TAKE PART IN THIS STUDY?

Taking part in this study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your medical care. You can still get your medical care from a hospital or clinic affiliated with Emory University.

If for any reason the study is stopped early, you doctor will discuss other treatment options with you. If you decide to stop participating in the study, please talk to your doctor first. Your doctor may ask you to have medical evaluations performed and blood drawn for safety reasons.

Your study doctor may remove you from the study, without your consent, if he/she does not consider it to be in your best interest to continue. This may occur if you experience an illness, research-related injury, or unacceptable side effects. You may also be withdrawn without your consent if you do not comply with the study requirements, such as problems keeping appointments for study procedures. It may also occur in response to new information being available about the study medication in relation to its safety or how effective it is. Your study doctor would also discontinue you from the study if you become pregnant. If you discontinue your participation in the study, the study doctor or one of the staff members will talk to you about any potential medical issues that may arise.

WHAT IF NEW INFORMATION ABOUT THIS TREATMENT IS LEARNED?

We may learn new things during the study that you may need to know. We will tell you about new information or changes in the study that may affect your health or your willingness to continue in the study. If so, you will be notified about any new information.

WHO CAN ANSWER MY QUESTIONS ABOUT THE STUDY?

You can talk to your study doctor about any questions or concerns you have about this study. Contact your study doctors Dr. John Kauh, at 404 778-2407 or Dr. Fadlo Khuri, at 404-778-4250.

For questions about your rights while taking part in this study, call the Chairman of the Emory University Institutional Review Board (a group of people who review

the research to protect your rights), Dr. James W. Keller, at (404) 712-0720.

WHERE CAN I GET MORE INFORMATION?

You may call the National Cancer Institute's Cancer Information Service at:

1-800-4-CANCER (1-800-422-6237) or TTY: 1-800-332-8615

You may also visit the NCI Web site at http://cancer.gov/

- For NCI's clinical trials information, go to: http://cancer.gov/clinicaltrials/
- For NCI's general information about cancer, go to http://cancer.gov/cancerinfo/
- For specific information regarding this study go to:

You will get a copy of this form. If you want more information about this study, ask your study doctor.

SIGNATURE

I have been given a copy of all pages of this form. I have read it or it has been read to me. I understand the information and have had my questions answered. I agree to take part in this study.

Participant	**Date**	**Time**
Person obtaining consent	**Date**	**Time**
Witness to signature	**Date**	**Time**
Principal Investigator (if different from above)	**Date**	

IRB#: 046-2006

Consent Form Approval Period

FROM: 12-28-06 TO: 12-27-07

AUTHORIZATION: db

General Information About the Collection and Use of Specimens for Research

You are being asked to allow samples of your bodily materials (tissue, cells, blood, serum or other material) to be collected and used in research. Such bodily materials are referred to as specimens and are very important in helping doctors and scientists learn more about caring for and treating people with cancer and other diseases. The use of specimens in scientific research can also help doctors and scientists understand why some people develop cancer and others don't, and why some people have cancers that respond or don't respond well to current therapies, for example.

The research that may be done with your specimens is not designed specifically to help you, but it may help others with cancer or other diseases in the future. Reports about research done with your specimens will not be given to you or your doctor, or be put in your health record. The research will not have an effect on your care.

If you agree to participate in this study, your specimens will be collected and used for the research described for this study. The next section of this consent will ask you to decide whether your specimens can be used for research.

Things to Think About
No matter what you decide to do, it will not affect your care.

If you decide now that your tissue can be used for research, you can change your mind at any time. If you do change your mind, contact the physicians responsible for carrying out this study, Dr. John Kauh or Dr. Fadlo R. Khuri, and let them know that you no longer want your specimens to be used. If necessary, staff at Emory Winship Cancer Center will destroy all of you specimens to make sure that they are no longer used for research.

To protect your privacy and confidentiality, the research investigators who study your specimens will never be given your name, address, phone number, Social Security number or any other personal information. In addition, your specimens will never be labeled with your name or other type of personal identifier.

Your tissue will be used only for research and will not be sold. The research done with your tissue may help to develop new treatments in the future; you should not expect to share in the profits of this. Your specimens will not be made available to researchers outside of Emory University.

The benefit of this research is that doctors may learn more about how to treat solid tumors.

Making Your Choices About Research

Please read each sentence below and think about your choice. After reading each sentence, circle "Yes" or "No". No matter what you decide to do, it will not affect your care. If you have any questions, please talk to your doctor, nurse or other type of healthcare provider.

You understand and agree that any tissue, blood, cell, or other biological samples that you provide as a participant in this research study are donations of these samples to Emory University. Upon your donation, these samples, and any data, discoveries, materials or other products that come from the samples, will be the exclusive, permanent property of Emory University. You will not have any property rights in the samples, nor will you have any property rights in or be entitled to compensation of any type for any products, data, or other items or information that is developed from the samples. Nevertheless, you may, at any time, request that Emory University destroy your sample, and Emory University will make all reasonable attempts to honor your request.

1. **Do you give permission for the clinical information collected by the Principal Investigator, Dr. John Kauh or Co-Principal Investigator, Dr. Fadlo R. Khuri, as part of your participation in this study to be used for research?**

   Please sign your name after you initial by your answers.

   Yes_____ **No**_____

Your signature: _____ Date: _____

Signature of Witness: _____ Date: _____

IRB#: 046-2006
Consent Form Approval Period
FROM: 12.28.06 TO: 12.27.07
AUTHORIZATION: db

YOUR PRIVACY

The privacy of your medical record is important to us. Before we start our research we want to tell you about a law that protects your medical record, and the information you give us for this study. The law is called the Health Insurance Portability and Accountability Act, or HIPAA for short.

Under HIPAA, your personal health information that identifies you receives greater protection. We will now tell you more about how we will protect your health information for this study.

WCI1106-05 A Phase I Biologic Study of Bexarotene (Targretin®) and Celecoxib in Patients with Solid Tumors Previously Treated with Standard Chemotherapy

John Kauh, M.D.

Authorization to Use or Disclose Health Information that Identifies You for a Research Study

Subject Name:_____ Study Number:_____

If you sign this document, you voluntarily give permission to the people or groups of people listed below to use or disclose (release) your health information that identifies you in connection with Research Study that is described below:

Research Study:

People That Will Use or Disclose Your Information and Purpose of Use/Disclosure: The following individuals or groups are people who will be conducting the Research Study or who have the job of monitoring and regulating research and who will use or disclose your health information to do this work (the "Information Recipients"):

Person/Entity	***Purpose***
Researchers	*To conduct the study entitled," The purpose of this study is to test the safety of the combination of bexarotene and celecoxib and see what effects (good or bad) it may have on your tumor, to determine the smallest dose that may lead to a shrinkage of your tumor, and to determine the highest dose of the combination of bexarotene and celecoxib that can be given without causing severe side effects.*
Governmental Agencies with oversight over the research being conducted, including the FDA and OHRP	*To monitor safety, efficacy and compliance with applicable laws and regulations.*
University personnel, committees and departments charged with oversight of research, including the IRB.	*To monitor safety and compliance with applicable laws, regulations and University policies and procedures.*
"" Corporation, the study sponsors.	*To provide oversight for the study and to perform data analysis.*
Statisticians hired by the study sponsor.	*To perform data analysis.*
Outside laboratories hired by the Department of Defense or Emory University.	*To analyze specimens.*
Clinical research organizations hired by the Department of Defense or Emory University	*To compile data and perform study monitoring.*
Study monitors hired by the Department of Defense or Emory University	*To verify that data has been properly collected for reporting to the FDA.*
Researchers working on this study at other sites, including sites at "" and the IRBs for those sites.	*To assist in the conduct of this study; for future research related to this study; and to report problems ("adverse events") and issues that occur during the study.*

By signing this document you agree to allow these Information Recipients to use or disclose your health information that identifies you for the Research Study, or to monitor or regulate research. In addition,

your health information may be used or disclosed as required by law, and it may be shared with a public health authority. that is authorized by law to collect or receive such information for the purpose of preventing or controlling disease, injury or disability and/or conducting public health surveillance, investigations or interventions.

Description of Health Information that will be used or disclosed
The Researchers and Regulators may use or disclose the following health information about you:

name, telephone numbers, address, all elements of dates, SSN, date of birth, medical records numbers, and Emory medical history information.

Revoking your Authorization:

You do not have to sign this Authorization. In addition, if you sign this Authorization, later, you may change your mind at any time and revoke (take back) this Authorization. If you want to revoke this Authorization you must write to:

John Kauh, M.D.
Emory University, Winship Cancer Institute
1365C Clifton Road, NE
Atlanta, GA 30322

Attached is pre-printed revocation letter to **Emory University** for your use.

If you revoke your Authorization, the Researchers will not collect any more health information that identifies you, but they may use or disclose information that you already gave them in order to notify any of the other Researchers that you have revoked your authorization; to maintain the integrity or reliability of the Research Study; and to comply with any law that they are required to obey.

Other Items You Should Know:

The Information Recipients who work for Emory University Winship Cancer Institute are required by HIPAA to protect your health information. However, some of the other Information Recipients who receive your health information do not work for Emory University Winship Cancer Institute, and they may not be required by HIPAA to protect your health information. These Information Recipients may share your information with others without your permission if the law permits them to do so.

You do not have to sign this authorization form, but if you do not, you may not participate in the Research Study or receive research-related treatment. You may still receive non-research related treatment.

If the Research Study involves medical treatment, then, in order to maintain the integrity of the research study, you generally will not have access to your personal health information related to this Research Study until the study is complete. When the study is complete, then, at your request, you may generally have access to any of your personal health information related to the research that makes up a part of the medical information and/or billing records that your health care providers use to make decisions about you. If access to this information is needed before the end of the Research Study for your treatment, then the information will be provided to your physician.

If your identifying information is removed from your health information, then the information that remains will not be subject to this authorization and it may be used or disclosed for other purposes.

Expiration Date: If you agree by signing this form that researchers can use your personal health information, this permission has no expiration date. However, as stated above, you can change your mind and withdraw your permission at any time.

As a study participant, if you any questions regarding the study, you may call Dr. John Kauh the study's Principal Investigator at (404) 778-2407. If you have any questions regarding your rights as a study subject, you may call Dr. James W. Keller, Chairman of the Emory University Institutional Review Board at (404) 712-0720.

A copy of this authorization form will be given to you. Also, a copy of this authorization may be placed in your medical record.

_____

Signature of Study Subject OR Subject's Legal Authorized Representative

_____ _____

Date Time

_____

Printed Name of Study Subject OR Subject's Legally Authorized Representative

Relationship to Study Subject: _____

_____

Signature of Person Obtaining Authorization

_____ _____

Date Time

Thank You for Your Participation

IRB#: 046-2006

Consent Form Approval Period

FROM: 12.28.06 **TO:** 12.27.07

AUTHORIZATION: dh